Dissecting the Role of Parp1/Artd1, Sox2 and Fgf4 during Reprogramming

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Abstract

The ground-breaking discovery of Takahashi and Yamanaka provided evidence that forced expression of four transcription factors, Oct4, Sox2, Klf4 and c-Myc, can reprogram mouse somatic cells to pluripotent stem cells, also known as induced pluripotent stem cells (iPSCs). Similar to embryonic stem cells (ESCs), iPSCs have the ability on one hand to permanently self-renew and on the other hand to differentiate into any cell type of the body. Thus, these cells offer great potential to develop disease models *in vitro*, drug and toxicity screening tools as well as patient-derived iPSCs for future clinical applications. Regarding these characteristics, it is not surprising that in the past years a variety of reprogramming methods have been developed. However, most of these approaches coincide with low reprogramming efficiency, the genomic integration of the reprogramming factors and a long duration of the reprogramming process. In addition, the understanding of the underlying molecular mechanisms is still limited. Therefore, increasing the knowledge of the processes that orchestrate the reprogramming process is crucial for the establishment of more efficient reprogramming techniques and the generation of high-quality iPSCs.

The key aim of this thesis was to gain knowledge of the molecular mechanisms driving the reprogramming process, in particular the role of Artd1, Sox2 and Fgf4 in the early phase of the reprogramming process.

Artd1/Parp1 is an abundant nuclear DNA-binding protein that catalyses the covalent attachment of poly(ADP-ribose) (PAR) to itself and to other nuclear acceptor proteins. ADP-ribosylation plays an important role in numerous biological processes, including maintenance of genomic integrity, cell death and transcriptional regulation. The transcription factor Sox2 is a key player in the transcriptional network maintaining the pluripotent state of ESCs. Moreover, it is one of the four factors that initiate the reprogramming process. The two proteins Artd1 and Sox2 have already been shown to cooperatively regulate the expression of *Fgf4* in ESCs. Fgf4 in turn is typically expressed in pluripotent stem cells such as ESCs and iPSCs. Furthermore, it is involved in the control of pluripotency and lineage specification of ESCs. Thus, we aimed to investigate the potential function of Artd1, Sox2 and Fgf4 during the reprogramming process. In an initial experiment we show that absence of Artd1 or inhibition of its enzymatic activity significantly reduces the reprogramming efficiency of mouse fibroblasts. A subsequent time-course experiment reveals that a functional enzymatic activity of Artd1 is essential during the first days of the reprogramming process in particular. We can show that in this early phase of reprogramming Artd1 interacts with and PARylates Sox2. As Artd1 and Sox2 have been demonstrated to regulate the expression of *Fgf4* in ESCs, we wondered whether the Sox2-Artd1 complex holds a similar role during reprogramming. By analysing the expression level of *Fgf4* in wild-type cells, Artd1-deficient fibroblasts and PARP-inhibitor treated wild-type fibroblasts and subse-

quent chromatin immunoprecipitation against Sox2, we demonstrate that ADP-ribosylation of Sox2 strengthens its binding to the *Fgf4* enhancer, thereby stimulating the transcription of *Fgf4*. The finding that inhibition of the Fgf receptor tyrosine kinase activity significantly reduced the reprogramming efficiency highlights the importance of functional Fgf4. Thus, we aimed to understand the mechanism by which Fgf4 modulates the reprogramming process. Our data illustrate that addition of exogenous Fgf4 during reprogramming induces an increase in the number of iPSC colonies and that inhibition of FGF-signalling negatively affects the reprogramming efficiency. This effect is most significant when blocking FGF-signalling between d4 and d8 of the reprogramming process. We finally demonstrate that in this phase of the reprogramming process, Fgf4 regulates cell proliferation and favours mesenchymal-to-epithelial transition.

Taken together, our work demonstrates that Artd1-mediated PARylation of Sox2 is essential to positively modulate *Fgf4* transcription during the reprogramming process. In addition, we identify Fgf4 as an important factor for promoting reprogramming by favouring mesenchymal-to-epithelial transition.

Zusammenfassung

Takahashi und Yamanaka machten im Jahre 2006 eine bahnbrechende Entdeckung, welche zeigte, dass die Expression der vier Transkriptionsfaktoren Oct4, Sox2, Klf4 und c-Myc ausreicht um somatische Zellen der Maus in pluripotente Stammzellen umzuwandeln (=umzuprogrammieren). Sie gaben diese Zellen den Namen „induzierte pluripotente Stammzellen“ (iPS Zellen). Ähnlich wie embryonale Stammzellen haben auch iPS Zellen die Fähigkeit sich dauerhaft selbst zu erneuern und sich in jede Gewebezelle unseres Körpers zu entwickeln. Dank dieser Eigenschaften weisen diese Zellen ein grosses Potential auf für die Entwicklung von *in vitro* Krankheitsmodellen, Drogen- und Toxizitätsscreening-Tools und Patienten-spezifischen iPS Zellen für klinische Anwendungen. Das Wissen über das Potential dieser Zellen hat dazu geführt, dass in den letzten Jahren viel Zeit in die Optimierung und Neuentwicklung von Reprogrammierungsmethoden investiert wurde. Dennoch haben die meisten dieser Methoden den Nachteil einer geringen Effizienz, einer langen Prozessdauer und der genomischen Integration der Reprogrammierungsfaktoren. Erschwerend kommt hinzu, dass das Wissen über die dem Reprogrammierungsprozess zugrunde liegenden molekularen Mechanismen noch immer sehr begrenzt ist. Daher ist es für die Entwicklung von effizienteren Reprogrammierungsmethoden und die Herstellung von hochwertigen iPS Zellen von entscheidender Bedeutung, mehr Kenntnisse über diese molekularen Mechanismen zu gewinnen. Dementsprechend war es das Ziel dieser Arbeit einen vertieften Einblick in die den Reprogrammierungsprozess steuernden molekularen Mechanismen zu erhalten. Es gelang schlussendlich aufzuzeigen, dass Artd1, Sox2 und Fgf4 in der frühen Phase des Reprogrammierungsprozesses eine wichtige Rolle spielen.

Das Enzym Artd1/Parp1 katalysiert die kovalente Bindung von Poly(APD-Ribose) an Akzeptor-Proteine und sich selbst im Zellkern. ADP-Ribosylierung spielt in vielen biologischen Prozesse eine wichtige Rolle, so zum Beispiel bei der Aufrechterhaltung der genomischen Unversehrtheit, beim Zelltod und bei der Regulation der Transkription. Das Protein Sox2 ist ein wichtiger Transkriptionsfaktor für die Erhaltung der Pluripotenz von embryonalen Stammzellen und gehört zu den vier Faktoren, die den Reprogrammierungsprozess initiieren. Es wurde bereits in embryonalen Stammzellen gezeigt, dass Artd1 und Sox2 gemeinsam die Expression von Fgf4 modulieren. In embryonalen Stammzellen reguliert Fgf4 die Entscheidung zwischen Erhaltung des pluripotenten Status oder Differenzierung zu spezifizierten Zellen. Interessanterweise wurde auch gezeigt, dass Fgf4 nicht nur in embryonalen Stammzellen, sondern auch in iPS Zellen exprimiert wird. Basierend darauf lag unser Interesse darin, die Funktion von Artd1, Sox2 und Fgf4 während des Reprogrammierungsprozesses zu untersuchen. Ein erster Versuch zeigte, dass die Abwesenheit von Artd1 oder die Hemmung der enzymatischen Aktivität von Artd1 die Reprogrammierungseffizienz von Fibroblasten signifikant reduziert. Mit einem nachfolgenden Experiment konnten wir demonstrieren, dass die enzymatische Aktivität von Artd1

nur in den ersten Tagen des Reprogrammierungsprozesses notwendig ist. In dieser frühen Phase des Prozesses interagiert Artd1 einerseits mit Sox2 und ist andererseits für die Poly(ADP-Ribosylierung) von Sox2 zuständig. Frühere Publikationen zeigten, dass in embryonalen Stammzellen diese Modifizierung von Sox2 entscheidend für die Expression von *Fgf4* ist. Daher haben wir uns die Frage gestellt, ob der Sox2-Artd1 Komplex auch während des Reprogrammierungsprozesses die Expression von *Fgf4* steuert. Durch eine Expressionsanalyse von *Fgf4* und einen Chromatinimmunpräzipitationsassay in Wildtypzellen, Artd1-/- Zellen und PARP-inhibierten Wildtypzellen konnten wir zeigen, dass die Bindung von Sox2 an den Enhancer von *Fgf4* durch die ADP-Ribosylierung begünstigt und verstärkt wird. Weiter konnten wir aufweisen, dass diese Bindung entscheidend ist für die Aktivierung der Transkription von *Fgf4*. Basierend auf diesen Resultaten untersuchten wir, ob Fgf4 selbst eine wichtige Rolle im Reprogrammierungsprozess spielt. Es zeigte sich, dass die Inhibierung der Tyrosin-Kinase-Aktivität des Fgf-Rezeptors zu einer signifikanten Reduktion der Reprogrammierungseffizienz führt. Auf dieser Grundlage entschieden wir uns die Rolle von Fgf4 während des Reprogrammierens näher zu analysieren. Unsere Resultate offenbarten einerseits, dass die exogene Zugabe von Fgf4 während dem Reprogrammierungsprozess zu einer signifikanten Zunahme der iPS Kolonien führt und andererseits, dass sich die Hemmung des FGF-Signalweges negativ auf die Reprogrammierungseffizienz auswirkt. Dieser von Fgf4 initiierte Effekt war zwischen dem Tag 4 und Tag 8 am stärksten sichtbar. Weiterführende Experimente demonstrierten schlussendlich, dass Fgf4 einerseits auf die Zellproliferation einwirkt und andererseits die mesenchymal-epitheliale Transition begünstigt.

Zusammenfassend kann gesagt werden, dass die durch Artd1 vermittelte PARylierung von Sox2 wichtig ist, um die Transkription von Fgf4 während des Reprogrammierungsprozesses zu fördern. Weiter gelang es uns nachzuweisen, dass Fgf4 die mesenchymal-epitheliale Transition unterstützt und deshalb ein wichtiger Faktor zur Förderung des Reprogrammierungsprozesses ist.

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Abbreviations

AP	Alkaline phosphatase
Artd1	ADP-ribosyltransferase diphtheria toxin-like 1
BMP4	Bone morphogenic protein 4
DNA	Deoxyribonucleic acid
ECCs	Embryonic carcinoma cells
EGCs	Embryonic germ cells
EMT	Epithelial-to-mesenchymal transition
Ep-Cam	Epithelial cell adhesion molecule
Erk1/2	Extracellular signal-related kinase ½
ESCs	Embryonic stem cells
Esrrb	Oestrogen-related receptor β
FBS	Fetal bovine serum
Fbx15	F-box only protein 15
Fgf4	Fibroblast growth factor
FgfR	Fibroblast growth factor receptor
GSK3β	Glycogen synthase kinase β
HSGAG	Heparan sulphate glycosaminoglycan
ICM	Inner cell mass
iPSCs	Induced pluripotent stem cells
Klf4	Krüppel-like factor 4
LIF	Leukemia inhibitory factor
MEFs	Mouse embryonic fibroblasts
mESCs	Murine embryonic stem cells
MET	Mesenchymal-to-epithelial transition
miRNA, miR-	microRNA
NAD+	Nicotinamide adenine dinucleotide
NT	Nuclear transfer
Ocln	Occludin
Oct4	Octamer-binding transcription factor 4
Parp1	Poly(ADP-ribose) polymerase 1
PARylation	Poly(ADP-ribosylation)
PD17	PD173074

Pou5f1	POU domain, class 5, transcription factor 1
Rex1	Reduced expression protein 1
RNA	Ribonucleic acid
Sall4	Sal-like protein 4
Sox2	SRY (sex determining region Y)-box 2
SSEA1	Stage-specific embryonic antigen 1
Stat3	Signal transducer and activator of transcription 3
STGCs	Syncytiotrophoblastic giant cells
TE	Trophectoderm
Tet2	Ten-eleven translocation-2
TgfβR	Transforming growth factor-β receptor
Wild-type	Wt
2nd MEFs	Secondary mouse embryonic fibroblasts

A. Introduction

Induced pluripotent stem cells (iPSCs) are pluripotent cells obtained through the reprogramming of somatic cells into a ground state of pluripotency. They show properties similar to embryonic stem cells (ESCs), including the ability of self-renewal for an indefinite period of time and the capacity to differentiate into cells of all three germ layers. These features harbour a huge potential for improving the understanding and treatment of diseases. In addition, patient-derived iPSCs provide a valuable source for a range of applications, including autologous cell therapy, managing degenerative diseases, repairing genetic defects and as substrates for drug, toxicity and therapeutic screens. However, the efficiency of reprogramming and the safety of the generated iPSCs remain considerable hurdles to overcome. Therefore, a better mechanistic understanding of the reprogramming process is critical for enhancing both the efficiency of iPSC generation and their safety for use in a clinical setting. In this work, the significance of the functional interaction of Sox2 (SRY (sex determining region Y)-box 2), Artd1 (ADP-ribosyltransferase diphtheria toxin-like 1; formerly called poly(ADP-ribose) polymerase 1 [Parp1]) and Fgf4 (fibroblast growth factor 4) during the early phase of the reprogramming process is examined.

1. Pluripotent stem cells

Stem cells are undifferentiated cells that are present in the embryonic, fetal and adult stages of life. They are defined by their ability to renew themselves and differentiate into various types of cells and tissues. Self-renewal is the process by which a stem cell divides symmetrically and asymmetrically. Through symmetrical replication, a stem cell gives rise to two similar daughter stem cells, whereas asymmetrical division results in one daughter cell that is identical to the original stem cell and another that differentiates into a specialized cell type (Shenghui et al., 2009). This capacity for self-renewal is essential for stem cells to expand their numbers during development and to maintain tissue homeostasis by replacing injured and aged cells. The other main characteristic of stem cells is differentiation, which describes the transition of a stem cell into a more specialized cell type that loses the ability to multiply itself and thus self-renew (Smith, 2001). Stem cells can be classified into five groups depending on their differentiation potential: totipotent, pluripotent, multipotent, oligopotent and unipotent (Table 1) (Kolios and Moodley, 2013).

Tab. 1: Classification of stem cells based on differentiation potential

Potency	Description	Examples
Totipotent	Can differentiate into embryonic and extraembryonic tissues	Zygote and early blastomeres of the mammalian embryo
Pluripotent	Can differentiate into all three germ layers (ectoderm, mesoderm, endoderm) of an organism	Embryonic stem cells Induced pluripotent stem cells
Multipotent	Can differentiate into multiple cell types of one single germ layer	Mesenchymal stem cells
Oligopotent	Can differentiate into cell types of two or more lineages within a specific tissue	Hematopoietic stem cells
Unipotent	Can differentiate into one cell type	Spermatogonial stem cells

These properties make stem cells valuable in a wide range of applications in biology and medical sciences. For example, they are used to study mammalian embryogenesis, to set up human models of diseases *in vitro*, in drug and toxicity screens, and also in cell therapy. Particularly pluripotent stem cells, such as ESCs and iPSCs, are an important tool as they harbour the ability to differentiate virtually into all cell types of the body. However, the prerequisite for using them on a regular basis for clinical application lies in a thorough and deeper understanding of molecular mechanisms regulating their pluripotency.

1.1. Embryonic stem cells

Murine embryonic stem cells (mESCs) were first isolated from the inner cell mass (ICM) of blastocyst stage embryos and grown *in vitro* in 1981 (Evans and Kaufman, 1981; Martin, 1981). Subsequent studies showed that mESCs contribute to all somatic cell lineages, including the germ line, after injection into a blastocyst stage host embryo (Bradley et al., 1984; Nagy et al., 1990). *In vitro,* mESCs can be propagated indefinitely in the undifferentiated state, but retain the ability to differentiate into all cell types of the body when induced by the appropriate signals. To maintain this pluripotent state *in vitro*, the appropriate culture conditions are of major importance: mESCs can be maintained by cultivating them on mitotically inactivated mouse embryonic fibroblasts (feeders) in a medium containing leukemia inhibitory factor (LIF) and fetal bovine serum (FBS) containing bone morphogenic protein 4 (BMP4) (Smith et al., 1988; Ying et al., 2003). Recently, it has been shown that small-molecule inhibitors of protein kinases ERK1/2 (extracellular signal-related kinase 1/2) and GSK3β (glycogen synthase kinase beta) can be used to replace the feeders, cytokines and serum (Ying et al., 2008). The initial isolation of mESCs in 1981 and the subsequent increase in knowledge of mESCs culturing, handling and directed differentiation paved the way for major breakthrough for developmental biology, genetic engineering and the generation of transgenic animals for studying various diseases. The generation of human ESCs in 1998 marked another milestone in stem cell research and raised hope for use of ESCs in future treatment of human diseases (Thomson, 1998).

2. Induced pluripotent stem cells

The capabilities of ESCs to divide indefinitely and to differentiate into all the cell types of an adult organism permit the study of embryonic development and cell differentiation, and offer much hope for regenerative medicine. However, the ethical issues associated with (human) ESCs, the impossibility to generate patient-specific ESCs and the lack of disease-specific ESCs are viable limitations in ESC research. Therefore, the generation of pluripotent stem cells directly from adult somatic tissue would widen the range of applications of these cells. Three major approaches have been demonstrated to reprogram somatic cells to a pluripotent state: somatic cell nuclear transfer into oocytes (Wilmut et al., 1997; Wakayama et al., 1998), cell fusion between pluripotent stem cells and somatic cells (Tada et al., 1997; Tada et al., 2001; Cowan et al., 2005), and introduction of defined transcription factors into somatic cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007b), The latter approach was first reported by Takahashi and Yamanaka in 2006, who illustrated how cell fates can be altered by the ectopic expression of transcription factors, resulting in the generation of pluripotent stem cells, so called induced pluripotent stem cells. This technology has drawn attention from both the public and scientific community because it avoids using embryonic material and enables the generation of patient- and disease-specific pluripotent stem cells. In the following chapters, an in-depth insight into the iPSC technology is provided.

2.1. History of induced pluripotent stem cells

The establishment of the iPSC technology is based on findings of the past 50 years in the field of reprogramming of somatic cells (Yamanaka, 2012). The three most important streams of research that influenced and facilitated the generation of iPSCs are outlined here.

2.1.1. Reprogramming by nuclear transfer

Nuclear transfer (NT) involves transplantation of the nucleus from a differentiated somatic cell into an enucleated, unfertilized oocyte. The newly constructed cell can give rise to either a cloned organism, if transferred into the uterus of a surrogate mother, or to genetically matched ESCs if explanted into culture (Figure 1). NT was first shown by Briggs and King in 1952, with the transfer of nuclei isolated from late stage embryos and tadpoles into enucleated frogs' oocytes (Briggs and King, 1952), resulting in swimming tadpoles. However, Briggs and King did not succeed in reproducing this finding with nuclei from more specialized cells or in generating normal adult frogs. A decade later, Gurdon succeeded in generating adult frogs by transferring the nucleus of differentiated intestinal cells into amphibian oocytes (GURDON, 1962a, 1962b). Dolly was the first mammal cloned from an adult cell by fusing an unfertilized oocyte with a mammary adult cells (Wilmut et al., 1997). One year later, the first mice were cloned by removing the nuclei from mouse oocytes and replacing it with the nuclei of somatic cells (Wakayama et al., 1998). At present, a wide range of species have been successfully

cloned (van Thuan et al., 2010), demonstrating that fully specialized cells contain all of the genetic information required for supporting the generation of an entire organism. Furthermore, these findings show that oocytes contain factors that can reprogram somatic cell nuclei.

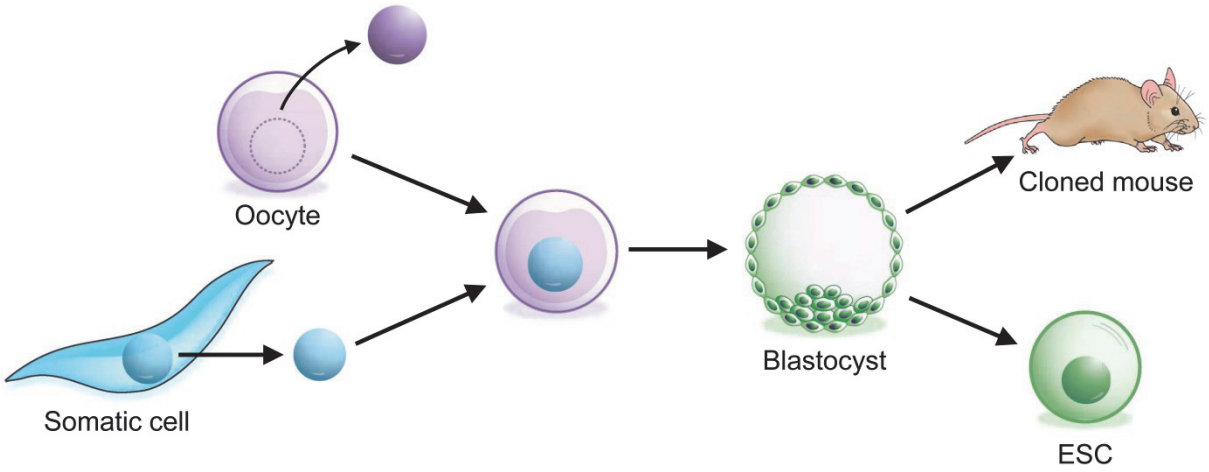

Fig. 1: Reprogramming by nuclear transfer

Nuclear transfer involves injection of the nucleus from a somatic cell into an enucleated oocyte. In the environment of the oocyte, the somatic cell nucleus is reprogrammed so that a blastocyst is generated. If transferred into the uterus of a surrogate mother, the blastocyst can give rise to a cloned organism (reproductive cloning). On the contrary, if explanted into culture it can give rise to ESCs (customized ESCs: therapeutic cloning) (modified from Yamanaka and Blau, 2010).

2.1.2. Reprogramming by fusion with pluripotent embryonic cells

Reprogramming of somatic cells has been demonstrated by fusion of somatic cells with ESCs (Figure 2), embryonic carcinoma cells (ECCs) (Stevens and Little, 1954) or embryonic germ cells (EGCs) (Matsui et al., 1992). In 1976, Miller *et al.* demonstrated that fusion of thymocytes with ECCs results in hybrid cells with the developmental properties of ECCs, in which the features of the somatic fusion partner were extinguished (Miller and Ruddle, 1976). Reprogramming of somatic cells was later obtained by electrofusion with EGCs (Tada et al., 1997) and mouse ESCs (Tada et al., 2001). In summary, all the embryonic cells (ESCs, ECCS and EGCs) have been shown to reprogram somatic cells to a pluripotent state, demonstrating that the pluripotent phenotype is dominant in such fusion products.

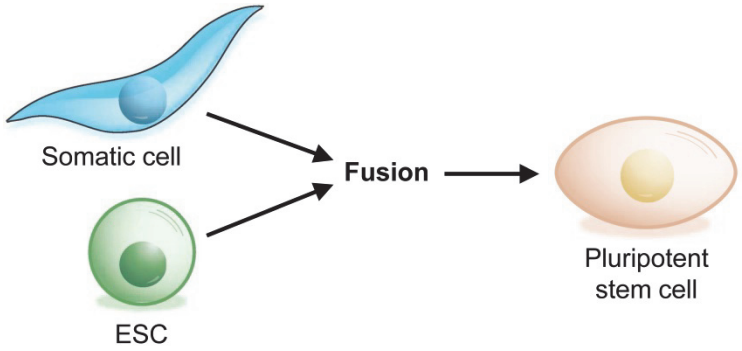

Fig. 2: Reprogramming by cell fusion

Fusion of somatic cells with ESCs results in the generation of hybrids that show all features of pluripotent stem cells (Yamanaka and Blau, 2010).

2.1.3. Conversion of one somatic cell type to another

The third finding that contributed to the establishment of the iPSC technology was the discovery of so called “master” transcription factors. The fate of a cell can be changed by the ectopic expression or ablation of lineage-associated transcription factors (master transcription factors), which normally regulate the expression of cell type specific genes and suppress the tissue-inappropriate genes during development. In mice, the first master transcription factors, namely MyoD, was identified by Lassar and colleagues in 1987 (Figure 3A). The ectopic expression of the skeletal muscle factor MyoD in fibroblasts induced the conversion of fibroblasts into myocytes (Davis et al., 1987). Subsequently, new master transcription factors were identified by various research groups (reviewed in (Graf and Enver, 2009), including the examples depicted in Figure 3.

These results show that transcription factors can directly convert cells from one lineage to another.

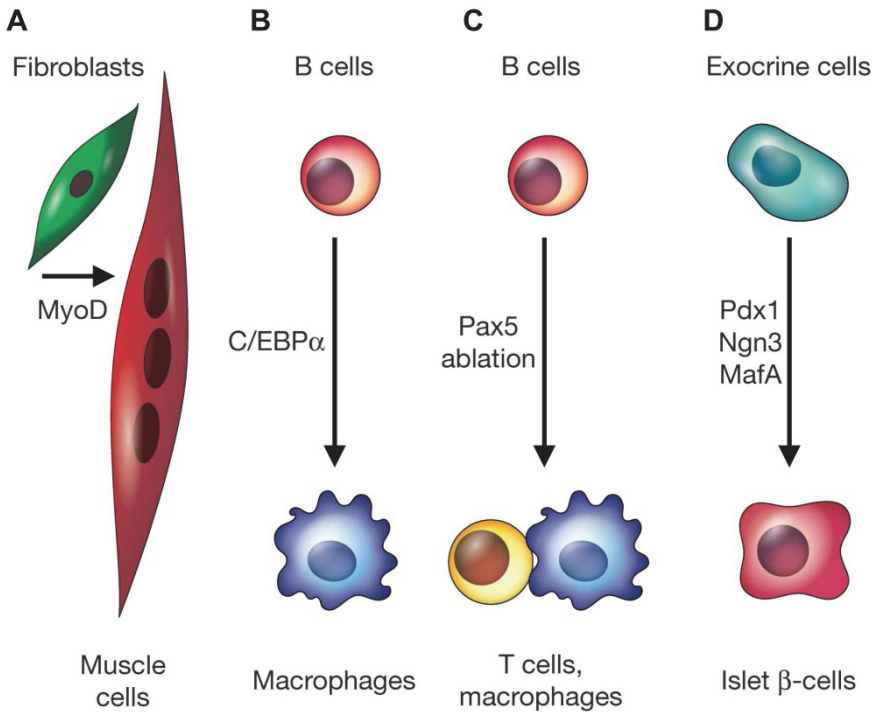

Fig. 3: Conversion of one somatic cell type to another

Examples of master transcription factor overexpression or ablation experiments that result in cell fate changes (modified from Graf and Enver, 2009). **A)** Retroviral vectors expressing MyoD induce myofibers in fibroblast cell lines (Davis et al., 1987). **B)** Primary B-cells can convert to functional macrophages by the expression of C/EBPα (Xie et al., 2004; Laiosa et al., 2006). **C)** The ablation of Pax5 in B-cells results in the generation of less specialized progenitors (Cobaleda et al., 2007). **D)** The combination of the three transcription factors Pdx1, Ngn3 and MafA enables the reprogramming of differentiated pancreatic exocrine cells into fully functional islet β-cells (Zhou et al., 2008). Abbreviations: MyoD: Myoblast determination protein 1, C/EBPα: CCAAT/enhancer binding protein alpha, Pax5: Paired box protein 5, Pdx1: Pancreatic and duodenal homeobox 1, Ngn3: Neurogenin 3, MafA: V-maf musculoaponeurotic fibrosarcoma oncogene homolog A.

2.2. Induction of pluripotent stem cells from somatic cells by defined factors

The findings presented in the last three chapters led Takahashi and Yamanaka (2006) to hypothesize that it is a combination of several factors in oocytes and ESCs that reprogram somatic cells back to the pluripotent state. To test this hypothesis, they selected 24 pluripotency-associated genes and developed an assay system in which the ability of the 24 genes to induce the pluripotent state could be detected as drug resistance. To do so, they integrated a reporter gene construct containing a neomycin resistance gene into the ESC-specific *Fbx15* (F-box only protein 15) locus of mouse embryonic fibroblasts (MEFs). The coexpression of all 24 genes from retroviral vectors activated *Fbx15* and induced the formation of drug-resistant colonies. The resulting colonies exhibited morphology similar to ESCs (Takahashi and Yamanaka, 2006). To determine which of the 24 candidates were critical to induce reprogramming in MEFs, individual factors were successively eliminated and finally a minimally required set of four genes, including *Oct4*, *Sox2*, *Klf4* and *c-Myc* (called hereafter Yamanaka factors), was identified (Figure 4). The iPSCs that were generated using the Yamanaka factors by selec-

tion for *Fbx15* activation expressed a majority of the marker genes for pluripotency, showed a high telomerase activity and stained positive for alkaline phosphatase (AP) activity. Furthermore, these iPSCs were able to generate teratoma after injection into immunocompromised mice and contributed to different tissues of developing embryos upon blastocyst injection (Takahashi and Yamanaka, 2006). However, the generated iPSCs showed global gene-expression patterns and DNA methylation status different from ESCs and were not able to generate adult or germline chimeras (Takahashi and Yamanaka, 2006). These Fbx15-selected iPSCs therefore appeared to be only partially reprogrammed. Soon after this study, selection for the reactivation of *Nanog* or *Oct4* instead of *Fbx15* resulted in germline competent iPSCs, which were molecularly and functionally closer to ESCs (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). A few years later, several iPSC lines have been identified that are capable of generating "all-iPSC" mice upon tetraploid complementation (Boland et al., 2009; Kang et al., 2009; Zhao et al., 2009; Stadtfeld et al., 2010), suggesting that the developmental potency of at least some iPSC clones is equivalent to ESCs.

One year after the generation of the first murine iPSCs, human iPSCs were obtained by inducing the overexpression of the same combination of factors (Takahashi et al., 2007b) or by using a different combination of factors, including OCT4, SOX2, NANOG and LIN28 (Yu et al., 2007).

Since the momentous finding of Takahashi and Yamanaka in 2006 and the generation of iPSCs from MEFs (Figure 4), many laboratories began to modify and refine the reprogramming method. This work by various groups supported the derivation of iPSCs from a number of different species and from other somatic cell populations (for reviews of the different methods, species and cell of origin see (Stadtfeld and Hochedlinger, 2010; González et al., 2011; Hussein and Nagy, 2012; Robinton and Daley, 2012).

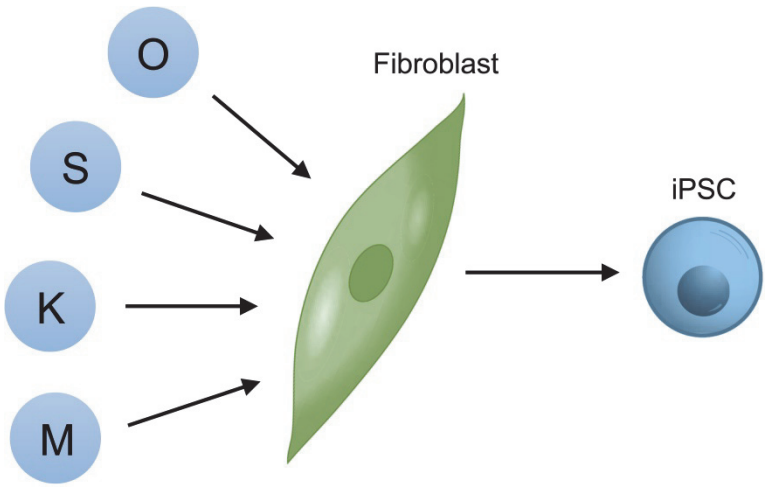

Fig. 4: Reprogramming by defined factors

Induction of pluripotent stem cells from somatic cells by defined factors. Transduction of somatic cells with the four transcription factors Oct4 (O), Sox2 (S), Klf4 (K) and c-Myc (M) leads to the formation of iPSCs, which have similar properties to ESCs.

2.3. Characteristics of murine induced pluripotent stem cells

Reprogramming of somatic cells to iPSCs is a highly inefficient process and only a fraction of resulting iPSCs show molecular and functional features comparable to ESCs. Therefore, analyses to accurately distinguish authentic iPSCs from those that are only partially reprogrammed are of major importance. The assays that were developed to test the pluripotency of ESCs serve hereby as a standard for the evaluation of iPSCs.

Assessing reprogramming starts with identifying colonies that display the typical morphology of pluripotent stem cells: In the mouse the colonies should grow in compact multilayers, have a round shape, exhibit a shiny and distinct border and are composed of cells with a large nucleus, large nucleoli and scant cytoplasma (Takahashi and Yamanaka, 2006). It has been shown that selection of iPSC colonies for picking and further expansion can be based solely on colony morphology (Meissner et al., 2007). Besides morphological criteria, analysis of molecular and functional attributes ascertain the pluripotent state of the iPSCs. On the molecular level, pluripotent iPSCs express the ESC-specific surface antigen SSEA1 (stage-specific embryonic antigen 1) and downregulate Thy1, which is expressed at high levels on the surface of fibroblasts and other differentiated cell types. In addition, they re-express endogenous pluripotency genes such as Oct4, Sox2 and Rex1 (Reduced expression protein 1) and stain positive for AP activity. Moreover, fully reprogrammed iPSCs reactivate the silent X chromosome in female cells during the late phase of the reprogramming process (Maherali et al., 2007; Wernig et al., 2007; Stadtfeld et al., 2008; Payer et al., 2011). Established iPSCs also express telomerase and re-establish a methylation pattern that is similar to the one from the ESCs (Takahashi and Yamanaka, 2006; Maherali et al., 2007; Wernig et al., 2007). If iPSCs acquire all these molecular features, they must be independent of transgene expression and therefore lack the expression of the delivered reprogramming factors (Takahashi and Yamanaka, 2006). At a functional level, pluripotent iPSCs must demonstrate the ability to differentiate into cells from all three germ layers. Characterization of the developmental potential of iPSCs begins with *in vitro* differentiation. The iPSCs can be differentiated by forming embryo-like aggregates called embryoid bodies or through two-dimensional directed differentiation after induction with specific factors. These differentiated cells are assayed for the expression of cell-type specific markers of each of the three germ layers. Another test involves *in vivo* differentiation in which iPSCs are either injected into the kidney capsules, testis or subcutaneously into the skin of an immune-deficient mouse and subsequently show the formation of teratoma consisting of tissues from all the three germ layers (Martin, 1981; Jaenisch and Young, 2008). Further analysis of the pluripotency of iPSCs involves the generation of chimaeras and the ability of chimaeras to produce all-iPSC mice in their offspring (germline transmission). The most rigorous test for pluripotency is the tetraploid complementation assay. In this assay, iPSCs are injected into a 4n host blastocyst, which results in animals exclusively derived from the iPSCs while the

extra-embryonic tissues are exclusively derived from the tetraploid cells. This test has been accomplished for only a limited number of iPSCs, but with an efficiency that correlates with the tetraploid complementation implemented with ESCs (Eakin et al., 2005; Zhao et al., 2009; Stadtfeld et al., 2010).

All these assays used to identify and evaluate iPSCs are summarized in table 2. A standardized and detailed analysis of iPSCs is extremely important, especially when anticipating the use of iPSCs for therapy.

Tab. 2: Characteristics of fully reprogrammed murine iPSCs

Morphology	Tight, rounded and multi-layer colonies with distinct boarders Display large nucleus, large nucleoli and scant cytoplasma
Cell surface antigens	Expression of SSEA1 Downregulation of Thy1
Pluripotency genes	Oct4 Nanog Sox2 Rex1
Enzymatic activities	AP Telomerase
XX status	Reactivate silent X chromosome
Methylation pattern	Similar to ESC pattern
Transgene expression	Silenced
***In vitro* differentiation**	Differentiate in the culture dish to various cells types Form embryoid bodies
***In vivo* differentiation**	Teratoma formation Chimera formation Germline transmission Tetraploid complementation

2.4. The 4 reprogramming factors

The induction of reprogramming in murine somatic cells was originally achieved by introducing the four transcription factors Oct4, Sox2, Klf4 and c-Myc into mouse fibroblasts (Takahashi and Yamanaka, 2006). After the introduction of the four reprogramming factors, the cells were continuously cultivated on feeders and in the presence of LIF. After 15 days, the cells showed an ES-cell like morphology and behaviour. In the following years, the Yamanaka protocol was constantly refined and the Yamanaka factors were combined with and/or replaced by various other factors (reviewed by (Hussein and Nagy, 2012), searching for the best and most efficient reprogramming cocktail for the respective cell type.

In our study we used the original Yamanaka factors and they are, therefore, described in the following sections.

2.4.1. Oct4

Oct4 (Octamer-binding transcription factor 4), also known as Pou5f1 (POU domain, class 5, transcription factor 1), is a member of the octamer-binding subgroup of the POU family of transcriptional factors. It plays an important role during the early embryonic development and in the induction and maintenance of cellular pluripotency.

During mouse development, Oct4 is expressed in the unfertilized egg and in all nuclei of the morula cells. Subsequently, the expression is reduced in the trophectoderm (TE) and becomes restricted to the ICM. In the post-implantation embryos, Oct4 is localized in the epiblast but disappears as cells undergo differentiation, with expression persisting in the primordial germ cells (Palmieri et al., 1994). The importance of Oct4 during early embryogenesis was highlighted by the fact that *Oct4*-deficient embryos die *in utero* at pre-implantation stages due to the absence of the ICM (Nichols et al., 1998). In ESCs, the expression of *Oct4* is required for acquisition of pluripotency and requires tight regulation. An expression above the critical level promotes differentiation into primitive endoderm and mesoderm, whereas repression of Oct4 causes loss of pluripotency and differentiation into trophectodermal tissue (Niwa et al., 2000). In addition, Oct4 belongs together with Sox2 and Nanog to the core transcriptional network, regulating the maintenance of pluripotency in human and mouse ESCs. Other transcription factors such as Sall4 (Sal-like protein 4) and Stat3 (Signal transducer and activator of transcription 3) are integrated into this pluripotency network and act in concert with the three core factors to activate targets that maintain pluripotency or repress factors that drive differentiation (Jaenisch and Young, 2008).

During the reprogramming process, Oct4 induces a subset of events in the early phase, regulates the transition towards pluripotency and maintains the ground state of pluripotency (Golipour et al., 2012). In addition, Oct4 supports the reorganization of the chromatin structure by recruiting chromatin remodelling complexes to the regulatory regions and by binding to closed chromatin states, thus acting as a pioneer transcription factor (Soufi et al., 2012; Esch et al., 2013). Therefore, Oct4 is generally considered to be an essential component in all reprogramming mixtures. For example, Oct4 cannot be replaced by any member of its protein family in the reprogramming cocktail, whereas the other three factors could be substituted by other members of their respective protein families (Ho et al., 2011). Furthermore, in combination with small molecules, Oct4 was sufficient to induce the reprogramming process in mouse fibroblasts (Li et al., 2011; Yuan et al., 2011). In 2010, Heng *et al.* succeeded in replacing Oct4 in the reprogramming cocktail by the orphan nuclear receptor Nr5a2 (Nuclear receptor subfamily 5 group A member 2). However, Nr5a2 functions as an upstream regulator of Oct4 by binding its enhancer and promoter and, therefore, regulates its expression (Heng et

al., 2010). Recently, several studies have demonstrated the generation of iPSCs without the transgenic expression of *Oct4*. Redmer *et al.* (2011) demonstrated that E-cadherin in combination with Sox2, Klf4 and c-Myc can replace the requirement for Oct4 during the reprogramming. The iPSCs generated in the presence of E-cadherin instead of Oct4 showed all the characteristics of pluripotent, reprogrammed cells (Redmer et al., 2011). Furthermore, Gao *et al.* (2013) showed that the DNA hydroxylase Tet1 promotes the demethylation and reactivation of Oct4 and is, therefore, able to replace Oct4 in the reprogramming cocktail (Gao et al., 2013). Shu *et al.* (2013) demonstrated that mesendodermal lineage specifiers in combination with Sox2, Klf4 and c-Myc are able to substitute Oct4 by repressing ectodermal genes (Shu et al., 2013). In addition, reprogramming has also been achieved solely by Nanog through activation of Sonic Hedgehog signalling (Moon et al., 2013) or by using only small-molecule compounds (Hou et al., 2013), demonstrating that Oct4 (and the other factors) can be replaced.

In summary, Oct4 plays an important role both during mammalian development and in the transition to and maintenance of pluripotency.

2.4.2. Sox2

Sex determining region Y (SRY) -box 2 (Sox2) is a member of the SRY-related high mobility group-box family of transcription factors. *Sox2* is expressed at all stages, from the oocyte to the blastocyst, of mouse preimplantation development (Keramari et al., 2010). At the blastocyst stage, Sox2 is initially present in both the ICM and the TE, but is later restricted to the ICM (Avilion et al., 2003). After gastrulation of the embryo, *Sox2* expression becomes restricted to the presumptive neuroectoderm, gut endoderm and primordial germ cells (Avilion et al., 2003; Yabuta et al., 2006). In the adult, Sox2 is present in proliferating cells in the central nervous system (Ellis et al., 2004).

The importance of Sox2 during early embryonic development was shown by the fact that zygotic depletion of Sox2 results in embryonic lethality after implantation caused by the failure to form the epiblast (Avilion et al., 2003). Later studies showed that maternal Sox2 accumulates in the cytoplasma of the oocytes and persists in all cells at least until the blastocyst stage. Interestingly, the depletion of maternal and zygotic *Sox2* transcription arrested the embryos at the morula stage and caused a failure to form TE. These results suggest that Sox2 is crucial for the segregation of the TE and ICM (Keramari et al., 2010). Consistent with the role of Sox2 in early embryogenesis, the derivation of ESCs from the ICM or the generation of trophoblast stem cells from the TE of *Sox2*-deficient embryos is not possible (Avilion et al., 2003). In ESCs, Sox2 plays an important role, together with Nanog and Oct4, in the gene regulatory network involved in maintaining their pluripotency (Boyer et al., 2005; Loh et al., 2006). Sox2 and Oct4 have been shown to interact and cooperatively regulate their own expression and the transcription of Sox2:Oct4 target genes, such as *Nanog*, *Lefty1, Utf1* and *Fgf4* (Dailey et al., 1994; Yuan et al., 1995; Nishimoto et al., 1999; Kuroda et al., 2005; Okumura-Nakanishi

et al., 2005; Nakatake et al., 2006). It has been shown, however, that the elevation of Sox2 levels in ESCs inhibits the expression of its own gene and the Sox2:Oct4 target genes, including *Oct4*, *Nanog* and *Fgf4* (Boer et al., 2007) and triggers the differentiation of ESCs into cells of various lineages, such as neuroectoderm, mesoderm and trophectoderm (Kopp et al., 2008). In contrast, deletion of Sox2 in ESCs promotes their differentiation into trophectoderm-like cells (Chew et al., 2005). Together, these data show that Sox2 plays important and often dosage dependent roles in the maintenance of ESCs and during early embryonic development.

During the reprogramming process, Sox2 acts together with Oct4 to activate common target genes, such as *Fgf4*, *Sall4* and *Fbx15*, that aid in the induction of pluripotency (Schmidt and Plath, 2012). Recently, we showed that poly(ADP-ribosylation) (PARylation) of Sox2 favours its binding to the *Fgf4* enhancer in the early phase of the reprogramming thereby activating *Fgf4* expression, which is crucial for an efficient reprogramming process (Weber et al., 2013). Furthermore, it has been shown that activation of the endogenous *Sox2* locus in the late phase of the reprogramming process initiates a series of consecutive steps leading to the activation of many pluripotency genes (Buganim et al., 2012). Sox2, however, can be replaced by the most closely related Sox family members (Nakagawa et al., 2008). In addition, several small-molecules have been identified that are able to replace Sox2 in reprogramming. Ichida *et al*. (2009) analysed 800 compounds for their ability to replace Sox2 in the reprogramming cocktail. They ended up with a transforming growth factor-β receptor 1 (TgfβR1) kinase inhibitor (RepSox), which was able to induce iPSCs in the absence of Sox2. Interestingly, this compound does not act by inducing *Sox2* expression. Instead, it enables reprogramming by inducing *Nanog* transcription (Ichida et al., 2009). Similar results were obtained by Maherali and Hochedlinger (2009), which showed that use of RepSox could enhance the reprogramming efficiency and replace Sox2 and c-Myc in the reprogramming cocktail (Maherali and Hochedlinger, 2009). Shi e*t al.* (2008) screened known drugs in their study to identify small molecules that can enable the reprogramming of MEFs to iPSCs solely in the presence of Oct4 and Klf4. Through the different screens, they found that a combination of a G9a histone methyltransferase inhibitor (BIX) (Kubicek et al., 2007) and a L-channel calcium agonist (BayK) (Schramm et al., 1983) was able to enhance the efficiency and replace Sox2 (Shi et al., 2008). Recently, a study demonstrated that ectodermal lineage specifiers in combination with Oct4, Klf4 and c-Myc are able to substitute Sox2 by repressing mesendodermal genes (Shu et al., 2013).

2.4.3. Klf4

Krüppel-like factor 4 (Klf4) belongs to the family of krüppel-like zinc finger transcription factors. *Klf4* is usually expressed in various tissues that have a high rate of cell turnover, including gut, skin and testis. In these tissues, Klf4 is primarily localized to the mitotically inactive population of cells (Gar-

rett-Sinha et al., 1996; Shields et al., 1996; Behr and Kaestner, 2002). *Klf4*-deficient mice die shortly after birth due to loss of epidermal barrier function as shown by a rapid loss of body fluid (Segre et al., 1999). In addition, various studies have shown that conditional deletion of *Klf4* affects many physiological functions, depending on the promoter: mutant mice show altered proliferation and differentiation of the gastric epithelium (Katz et al., 2005), corneal epithelial fragility, stromal edema and loss of conjunctiva goblet cells (Swamynathan et al., 2007), and altered proliferation and differentiation of vascular smooth muscle cells (Yoshida et al., 2008). Klf4 is also involved in further physiological processes, such as cell cycle-control, transcriptional regulation and apoptosis (Chen et al., 2001; Chen et al., 2003; Birsoy et al., 2008). Its capacity to regulate these diverse processes is through acting as a transcription factor. For example, Klf4 influences the cell cycle by activating numerous genes that encode negative regulators of the cell cycle as well as suppresses the transcription of genes that promote cell cycle progression (Chen et al., 2003). Because Klf4 has an anti-proliferative effect, it seems logical that Klf4 might function as a tumour suppressor. Indeed, various studies have demonstrated that the expression of *Klf4* is down-regulated in human cancers, including colorectal cancer (Zhao et al., 2004; Xu et al., 2008), gastric cancer (Wei et al., 2005; Kanai et al., 2006), esophageal cancer (Luo et al., 2004), bladder cancer (Ohnishi et al., 2003) and leukemia (Kharas et al., 2007). In support of the tumour suppressor role, studies using a conditional *Klf4*-deficient mouse specific for gastric epithelium demonstrate that absence of Klf4 results in increased proliferation and altered differentiation in the stomach leading to precancerous changes (Katz et al., 2005). Despite all the studies supporting the tumour suppressor nature of Klf4, there have been several reports suggesting that Klf4 might also act as an oncogene in certain contexts. Pandya *et al.* (2004) demonstrated that increased nuclear expression of *Klf4* in ductal breast carcinoma is associated with a more aggressive phenotype and a poorer prognosis (Pandya, 2004). Similar observations showed that overexpression of *Klf4* in the skin results in hyperplasia and dysplasia, eventually leading to squamous cell carcinoma of the skin (Foster et al., 2005; Huang and Liu, 2005). Whether Klf4 acts as a tumour suppressor or an oncogene may depend on the expression patterns of other genes, the chromatin environment of individual cells and the differences in cell context (Rowland et al., 2005; Rowland and Peeper, 2006).

In addition to its well-known expression in various tissues, Klf4 is highly expressed in undifferentiated ESCs, with its expression decreasing during differentiation (Bruce et al., 2007). In addition, overexpression of *Klf4* maintained ESCs in a self-renewal state, even in the absence of LIF. The same study also showed that ESC differentiation induced by Klf4 knockdown can be rescued by the overexpression of Nanog. This suggests that Klf4 is an upstream regulator of Nanog and this hypothesis was further proven by the results that Klf4 is able to bind to the Nanog promoter to regulate its expression (Zhang et al., 2010). Klf4 was shown to bind the Nanog promoter in ESCs in cooperation with

Oct4 and Sox2 (Wei et al., 2009). This complex of Oct4/Sox2/Klf4 seems to play an important role not only in ESCs maintenance, but also in the reprogramming of somatic cells to iPSCs. As one of the four Yamanaka factors, it has been suggested that Klf4 interacts with Sox2 and Oct4 during the reprogramming process. Profiling of Oct4, Sox2 and Klf4's DNA binding in the whole genome in iPSCs showed that these three factors colocalize on promoters of essential target genes required for reprogramming (Sridharan et al., 2009). The important role of Klf4 in this complex was further demonstrated by Wei *et al.* (2009): Mutant forms of Klf4, which interact with Oct4 and Sox2 but lack DNA binding activity, significantly reduce reprogramming efficiency (Wei et al., 2009). Another study demonstrated that target genes of Klf4 changed their expression both early and late in the reprogramming process. In the beginning, Klf4 repressed the expression of somatic genes, including *Tgfb1* and *Col6a1*, and in the last phase it activated the expression of pluripotency genes, such as *Oct4* and *Klf5* (Polo et al., 2012). Although these studies suggest an important role of Klf4 during the reprogramming process, several factors were shown to replace Klf4. Klf4 can either be replaced by family members, including Klf1, Klf2 and Klf5 (Nakagawa et al., 2008), or by the orphan nuclear receptor Esrrb (oestrogen-related receptor β) (Feng et al., 2009).

In summary, Klf4 is a transcription factor expressed in various tissues, which is important for many different physiologic processes, including cell cycle regulation and normal tissue homeostasis. In addition, Klf4 is able to either activate or repress target genes and can also function as an oncogene or a tumour suppressor, depending on the type of cancer. In addition, Klf4 plays an important role in ESCs maintenance and the induction of pluripotency.

2.4.4. c-Myc

The proto-oncogene c-Myc is a helix-loop-helix/leucine zipper transcription factor and belongs to a family that includes L-Myc and N-Myc. The c-Myc protein is involved in a broad range of biological processes, including the cell cycle, apoptosis, transcriptional regulatory mechanisms, stem cells and cancer (Meyer and Penn, 2008).

The expression of c-Myc has been shown to be absent in quiescent cells, but is induced upon the addition of growth factors. The expression peaks few hours after growth factor stimulation and decreases to a basal level needed in cycling cells (Kelly et al., 1983). The expression of c-Myc in cycling cells is crucial for G0/G1 to S phase progression and enables a shortened G1 phase (Steiner et al., 1995; Facchini and Penn, 1998). In addition, *c-Myc*-deficient immortalized rat fibroblasts showed prolonged cell doubling time and accumulated in the G1 and G2M phases of the cell cycle (Mateyak et al., 1997). Besides its function in driving the cell cycle, c-Myc is also involved in tumourigenesis and is shown to be overexpressed in a large percentage of human tumours, including cancers of lymphoid, mesenchymal and epithelial origin (Evan and Littlewood, 1993; Henriksson and Lüscher, 1996). The deregulation of *c-Myc* expression in human cancers is caused by various mechanisms, including

insertional mutagenesis, chromosomal translocation and gene amplification (Meyer and Penn, 2008). For example, Burkitt lymphoma, an aggressive B-cell lymphoma, results from chromosomal translocation that involves the *c-Myc* gene on chromosome 8 and the chromosomes 2, 14 and 22, which harbour the immunoglobin heavy and light chain genes (Dalla-Favera et al., 1982). After the role of c-Myc in cell proliferation and cell transformation had already been demonstrated, several studies in the early 1990's further demonstrated that ectopic expression of *c-Myc* drives the cells towards apoptosis. Deregulated *c-Myc* expression induced proliferation, but cell numbers did not necessarily increase, due to cell death by apoptosis (Evan et al., 1992; Shi et al., 1992). Furthermore, it has been shown that cells deficient in c-Myc are resistant to apoptotic stimuli (Soucie et al., 2001; de Alborán, I Moreno et al., 2004). Subsequent studies have demonstrated that multiple pathways regulated by c-Myc are responsible for fulfilling this biological activity (Dang et al., 2005; Nieminen et al., 2007). Two studies in parallel provided evidence that c-Myc deregulation activates the tumour suppressor p53 and therefore triggers apoptosis (Hermeking and Eick, 1994; Wagner et al., 1994). Another publication demonstrated that deregulated c-Myc upregulates $p19^{ARF}$ (Cyclin-dependent kinase inhibitor 2A, isoform 4), which subsequently activates p53 to regulate numerous target genes involved in apoptosis and growth arrest (Zindy et al., 1998). In short, c-Myc is able to sensitize cells to undergo apoptosis. Abrogation of the pro-apoptotic property profoundly contributes to tumourigenesis. c-Myc has also been shown to play a role in the promotion of proliferation and survival of somatic and embryonic stem cells. Wilson *et al.* (2004) demonstrated that c-Myc is essential in controlling the balance between hematopoietic stem cell self-renewal and differentiation, as well as being essential for proliferation of lineage-committed hematopoietic cells *in vivo* (Wilson et al., 2004). In ESCs, Cartwright *et al*. (2005) showed that the *c-Myc* gene is a direct transcriptional target of LIF/Stat3 signalling. Furthermore, they showed that ESCs express elevated levels of c-Myc and that, after LIF withdrawal, the c-Myc levels rapidly reduce during the early stages of differentiation upon GSK3β dependent degradation. In addition, forced expression of *c-Myc* in ESCs relieved the dependency of ESCs on LIF, whereas the dominant negative form of c-Myc induced differentiation even in the presence of LIF (Cartwright et al., 2005). Apart from its role in somatic and embryonic stem cells, c-Myc was also shown to be one of the four Yamanaka factors. One year after the first iPSC generation, Okita *et al.* (2007) showed that iPSC-derived animals develop tumours due to the reactivation of the *c-Myc* transgene. This observation represented a major safety concern regarding clinical applications of iPSCs (Okita et al., 2007). One year later, two studies demonstrated that c-Myc is dispensable when Oct4, Sox2 and Klf4 are used for the reprogramming, although with dramatically reduced efficiency (Nakagawa et al., 2008; Wernig et al., 2008). In their study, however, Nakagawa *et al.* (2008) also showed that fibroblasts express *c-Myc* endogenously and that the expression increases during the reprogramming process (Nakagawa et al., 2008), suggesting that endogenous c-Myc still participates

in reprogramming, albeit with lower efficiency. Another point to keep in mind is that the other family members, N-Myc and L-Myc, can be used as substitutes in the reprogramming cocktail (Nakagawa et al., 2008; Nakagawa et al., 2010), taking into account that the iPSCs that have been generated without c-Myc have already been selected for high levels of endogenous L- and N-Myc (Blelloch et al., 2007; Nakagawa et al., 2008). Overall, it appears clear that some *c-*, *N-* or *L-Myc* expression is required during reprogramming and that c-Myc enhances the reprogramming efficiency. The capacity of c-Myc to facilitate the reprogramming process could be due to its ability to promote proliferation, which in turn could indirectly alter the state of the cell or activity of the reprogramming factors. This hypothesis is supported by the finding that c-Myc plays a role in modifying chromatin structure to silence genes associated with differentiation (Knoepfler et al., 2006). Another explanation for the role of c-Myc during the reprogramming process is the finding that c-Myc has a large number of DNA binding sites in the genome (Knoepfler, 2007), including co-binding with Stat3 to regulatory elements of many pluripotency associated genes (Kidder et al., 2008).

Generally, c-Myc plays a role in various biological processes, including the cell cycle, apoptosis and cancer, and is involved in the induction and maintenance of pluripotency.

2.5. Mechanisms of the reprogramming process

In the past few years a variety of reprogramming methods have been developed to derive iPSCs, however, the majority of them struggle with a low efficiency, a long process length, the generation of iPSCs that vary in their developmental potential, and/or the genomic integration of the reprogramming factors (Jaenisch and Young, 2008; Ho et al., 2011; Robinton and Daley, 2012). Therefore, understanding the molecular mechanisms that underlie somatic cell reprogramming to pluripotency is crucial for the establishment of more efficient reprogramming techniques and the creation of high-quality iPSCs. It may also be useful for therapeutic applications.

The findings of various reports discussing the molecular mechanism of the reprogramming process revealed two major phases separated by an intermediate phase (Figure 5). After the induction of the cells with the Yamanaka factors (OKSM), a long stochastic phase of gene activation is initiated. At the end of the early stochastic phase, the cells enter an intermediate phase, which includes rate-limiting steps that reprogrammable cells must go through to enter the late hierarchical phase and become fully reprogrammed. In the following chapters, the three different phases are discussed, focusing on the early phase.

2.5.1. The early stochastic phase

In an immediate response to OKSM, the MEFs increase their proliferation rate after the first division and retain a rate similar to that observed in ESCs. In parallel, the cells show a decrease in size and also change their morphology from a fibroblastic shape to a small round ESC-like shape (Araki et al.,

2010; Smith et al., 2010). Apart from the morphological and the proliferation-based alterations, several changes can be observed on the molecular level during the early phase of the reprogramming process. Using an integrative genomic analysis, Mikkelsen *et al.* (2008) showed that the immediate response to OKSM is characterized by de-differentiation of MEFs and upregulation of proliferative genes. The de-differentiation is accounted by a decrease in the expression levels of mesenchymal genes normally expressed in MEFs. The effect on the proliferative response is assessed by upregulation of genes involved in DNA replication and cell cycle progression (Mikkelsen et al., 2008). In parallel, two other studies showed that the generation of iPSCs requires a mesenchymal-to-epithelial transition (MET) in the early phase of the reprogramming process (see chapter 3.1.) (Li et al., 2010; Samavarchi-Tehrani et al., 2010). Apart from their findings characterizing the early phase of the reprogramming, the study by Samavarchi-Tehrani *et al.* (2010) showed, using microarray analysis, that the process can be subdivided into three phases: initiation, maturation and stabilization. In our model (Figure 5) the initiation phase, marked by MET, is included in the early phase and the maturation and stabilization phases are linked to the late hierarchical phase and will be discussed later. Another study used genome-wide analyses to examine the molecular changes specific to cellular reprogramming. This study revealed two distinct waves of major gene activity and an intermediate period of reduced transcriptional change. The first wave occurred between days 0 and 3 and involved activation of genes responsible for cell proliferation, metabolism, cytoskeleton organization and downregulation of genes associated with development. Having a closer look at these transcriptional changes, the activated genes were divided into categories of genes that changed their expression in characteristic patterns. A large number of genes were immediately up- or downregulated after OKSM induction and then remained predominantly unchanged until the iPSC state. Upregulated genes with this transcriptional behaviour were largely involved in DNA replication and cell division processes, whereas downregulated genes were mainly engaged in cell adhesion and cell-cell contacts. Another group of genes, including pluripotency-associated gene such as *Fbx15*, *Sall1* and *Nr0b1*, showed a gradual upregulation after OKSM induction until the iPSC state. The second wave of major gene activity started after day 9, which is towards the end of the process (day 12) and therefore will be discussed later. Interestingly, cells resistant to successful reprogramming showed similar transcriptional changes in the first wave, however failed to undergo the second wave (Polo et al., 2012). In agreement with these results, a parallel study using quantitative proteomic analysis during the course of reprogramming demonstrated a two-step resetting of the proteome during the first 3 days and last 3 days (after day 12) of reprogramming. Proteins involved in regulation of gene expression, RNA processing and chromatin organization were strongly induced in the first 3 days. In addition, mitochondrial and metabolic proteins were elevated and proteins related to cell cycle and DNA repair were induced and maintained from day 3 onward until reaching of the iPSC state. Furthermore, epithelial markers such

as E-cadherin and Ep-Cam (Epithelial cell adhesion molecule) showed an increased expression after day 3. In contrast, the expression of extracellular matrix and cell adhesion proteins, as well as mesenchymal markers and matrix metalloproteases, was strongly reduced at day 3, reflecting the importance of MET in the early stage of reprogramming (Hansson et al., 2012). The majority of the thus far presented findings on the molecular mechanism underlying reprogramming was gained from population-based studies, which are essential for understanding the global changes during reprogramming. But as only a small fraction of the induced cells becomes reprogrammed and establishes a stable pluripotent state, population-based studies face difficulties in detecting changes in these rare cells determined to become iPSCs. In an attempt to overcome this problem, Buganim *et al.* (2012) performed two single-cell techniques to quantify the expression of 48 genes in single cells at various stages during the reprogramming process. The genes selected for analysis included those known to be involved in major events that occur during reprogramming. The selection comprises ESC-associated chromatin-remodelling genes and modification enzymes, due to the epigenetic changes during reprogramming. Because proliferation plays an important role during reprogramming, cell-cycle regulator genes were included as well. Furthermore, genes crucial for ESC maintenance and differentiation were selected, as well as a large number of pluripotency marker genes. Their analyses showed significant variations between sister cells of initial colonies, supporting the hypothesis that there is a stochastic gene activation after induction by OKSM (Buganim et al., 2012). These stochastic changes, in addition to the global changes such as alterations in genes associated with MET, proliferation and metabolism that must occur during reprogramming, are not restricted to cells that are destined to become iPSCs.

2.5.2. The intermediate phase

The early long stochastic phase is followed by an intermediate phase (Figure 5), which includes, among other things, an unknown rate-limiting event. This rate-limiting step is responsible for the low efficiency of the reprogramming process, as reprogrammable cells must pass this step in order to enter the late phase and become fully reprogrammed (Hanna et al., 2009; Buganim et al., 2012). In general, the transcriptional changes in the intermediate phase are small, but it has been shown that developmental-associated genes are transiently activated (Polo et al., 2012) and that glycolytic enzymes increase progressively, suggesting a gradual transformation of energy metabolism (Hansson et al., 2012). Another study by Buganim *et al.* (2012) demonstrated that pluripotency markers become stochastically activated during the intermediate phase. In some rare cases, this stochastic gene activation causes the activation of predictive indicators, including *Utf1*, *Esrrb*, *Dppa2* and *Lin28*, which mark cells that have a higher probability to instigate the late phase (Buganim et al., 2012).

2.5.3. The late hierarchical phase

The late hierarchical phase, including the maturation and stabilization phases, seems to be initiated by the activation of endogenous *Sox2* (Buganim et al., 2012; Golipour et al., 2012). Buganim *et al.* (2012) demonstrated that expression of endogenous *Sox2* appears late in the reprogramming process and is not activated in partially reprogrammed cells. Using a Bayes network model (a probabilistic model that represents a set of variables and their conditional dependencies), they further showed that activation of the endogenous *Sox2* locus triggers a series of steps of gene activation leading to the activation of many pluripotency genes (Buganim et al., 2012). The phase comprising the activation of *Sox2* until reaching the pluripotent state can be further divided into the maturation and stabilization phases (Samavarchi-Tehrani et al., 2010). Samavarchi-Tehrani *et al.* (2010) demonstrated that BMP stimulates the onset of *Nanog*, *Sall4* and endogenous *Oct4* expression, which marks the beginning of the maturation phase. Another study analysed the transition from the maturation to the stabilization phase, showing that silencing of OKSM transgenes is required for a successful transition (Golipour et al., 2012). Surprisingly, genes associated with gonads, gametes, cytoskeletal dynamics and signalling pathways were mainly upregulated during the transition phase and only few pluripotency regulators were involved. This finding suggests that different pathways play a role in the regulation of the transition to the stabilization phase and in the maintenance of pluripotency (Golipour et al., 2012). Genome-wide analyses revealed that genes associated with DNA binding and stem cell maintenance are upregulated in the late phase of the reprogramming process. The expression of these genes, including *Nanog*, *Oct4*, *Sox2*, *Esrrb* and *Dnmt3L*, mark the acquisition of a stable pluripotent state and the activation of the core pluripotency network (Polo et al., 2012). Quantitative proteomic analysis revealed that many proteins showing a reduced expression in the early phase (discussed above) were upregulated in the late phase, and vice versa. To specify, most of the mesenchymal markers, cell matrix-adhesion proteins and matrix metalloproteases were upregulated in the late phase, whereas epithelial markers started to decrease in expression. This finding suggests that an EMT (epithelial-to-mesenchymal transition)-like process is induced prior to reaching the pluripotent state. Interestingly, EMT was only detectable at the protein level and was not reflected at the transcript level, suggesting that this EMT-like process may be regulated at the posttranscriptional level (Hansson et al., 2012).

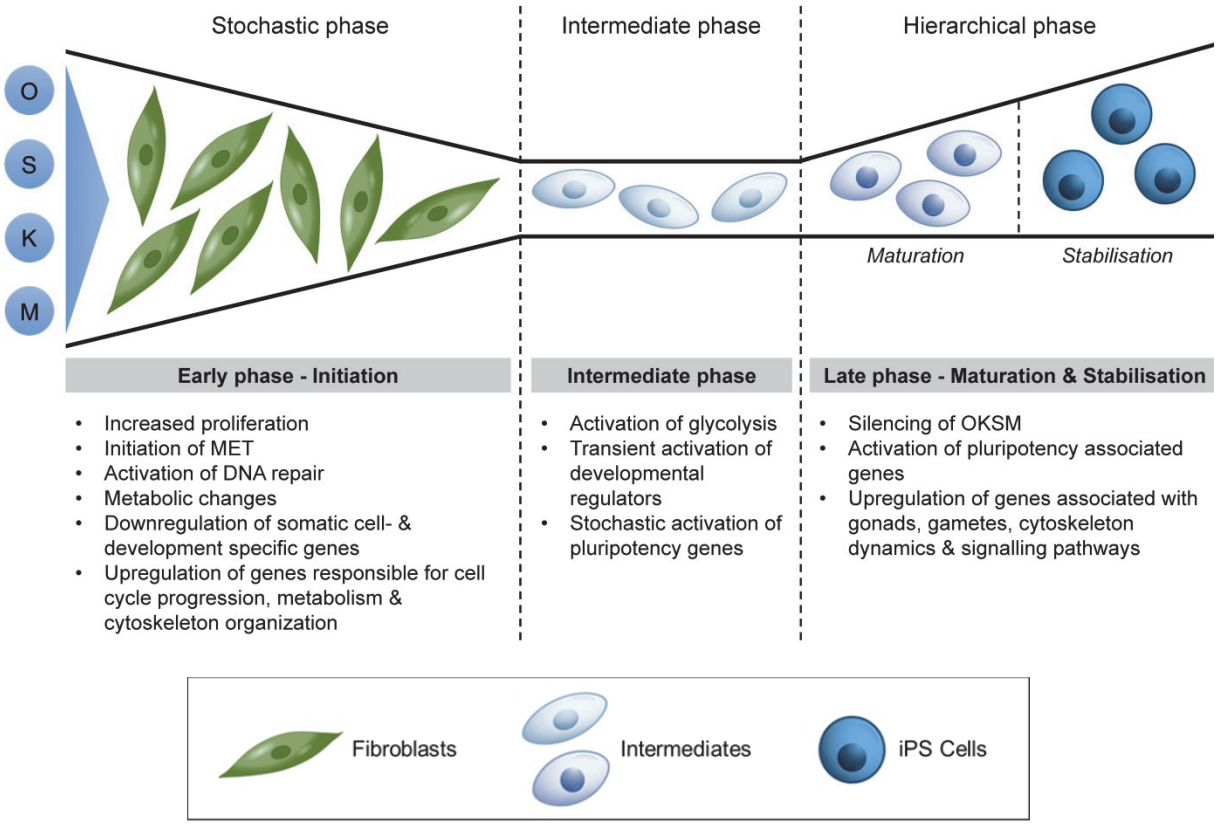

Fig. 5: Landmark events on the path to induced pluripotency

In the model discussed in this thesis, the reprogramming process can broadly be divided into two major phases separated by an intermediate phase. The expression of the four reprogramming factors Oct4 (O), Sox2 (S), Klf4 (K) and c-Myc (M) triggers the initiation of a long stochastic phase of gene activations. At the end of the early stochastic phase, the cells enter an intermediate phase, which includes rate-limiting steps that reprogrammable cells must go through in order to enter the late hierarchical phase and become fully reprogrammed. Known events occurring in the three phases of reprogramming somatic cells to iPSCs are shown in the table beneath the schematic of reprogramming (Buganim et al., 2013).

3. Epithelial-to-mesenchymal transition versus mesenchymal-to-epithelial transition

Epithelial-to-mesenchymal transition describes a form of cell plasticity, in which epithelial cells shed their epithelial characteristics and acquire the phenotype and behaviour of mesenchymal cells. During EMT, the cells lose their cell-to-cell contact and apical-basal polarity. Apart from losing their epithelial characteristics, the cells gain mesenchymal features involving end-to-end polarity and focal adhesion, resulting in increased cell motility and migratory and invasive capacity (Thiery et al., 2009). Mesenchymal-to-epithelial transition is the reverse process to EMT and describes the acquisition of epithelial characteristics and loss of mesenchymal features (Figure 6). The molecular and cellular mechanisms regulating EMT and MET are complex, as various transcription factors and signalling pathways are involved, depending on the physiological or pathological context (Thiery and Sleeman, 2006). Among the growth and differentiation factors that regulate EMT, Tgfβ represents a major and

potent inducer of EMT (Xu et al., 2009). The reverse process, MET, is therefore enhanced by the blocking of factors and signalling pathways that activate EMT. Additionally, BMPs and microRNAs (miRNA, miR-) can promote MET in a context-dependent manner (Gregory et al., 2008; Park et al., 2008; Samavarchi-Tehrani et al., 2010). EMT and MET are both connected with a switch in marker expression: EMT is characterized by the downregulation of epithelial markers, such as E-cadherin and Ep-Cam, as well as the upregulation of mesenchymal markers, including N-cadherin and the zinc finger transcription factors Snail and Slug. For MET the opposite occurs, namely the downregulation of mesenchymal markers and upregulation of epithelial markers (Thiery and Sleeman, 2006). Both processes are involved in early stages of development and in cancer metastasis (Funayama et al., 1999; Miyazawa et al., 2000; Locascio and Nieto, 2001; Thiery, 2002). Furthermore, EMT is required for tissue regeneration and wound healing. It is also associated with pathological stresses, including inflammation and organ fibrosis (Kalluri and Weinberg, 2009). On the contrary, MET, apart from its function in the formation of tissues and organs during development and in cancer metastasis, is involved in the generation of iPSCs (see chapter 2.5.1. and the following chapter).

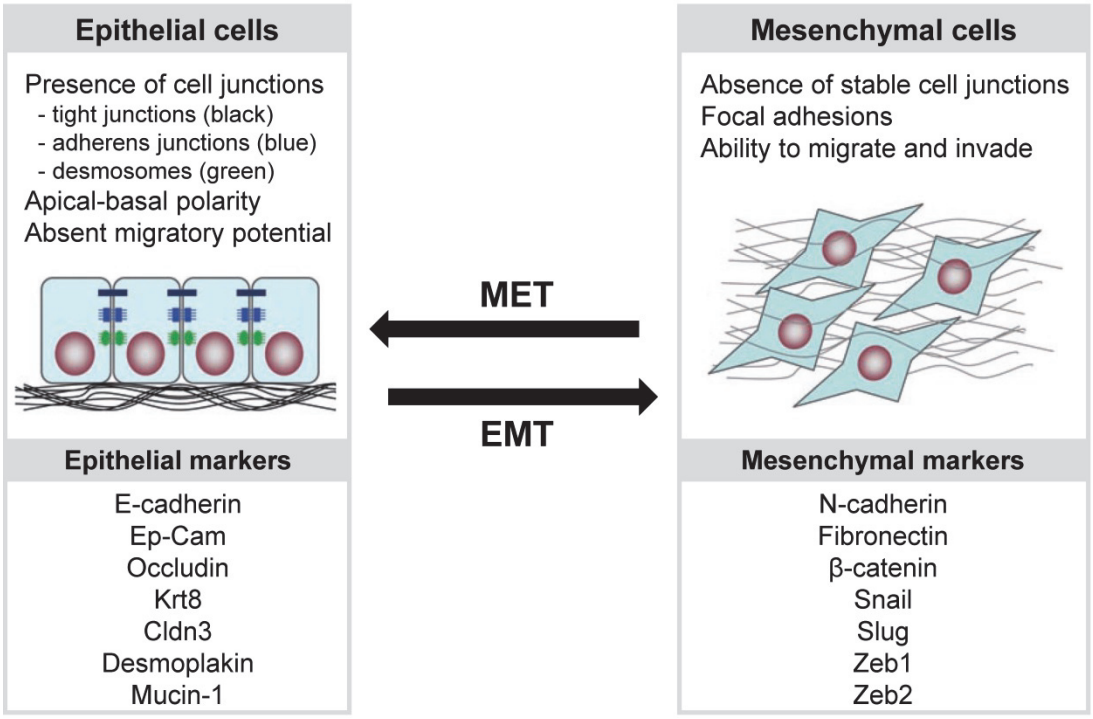

Fig. 6: Schematic representation of the EMT and MET

Epithelial cells contain specialized cell junctions, exhibit apical-basal polarity and show a limited potential for migration. In contrast, mesenchymal cells do not form specialized adhesion complexes, display focal adhesions and are able to migrate and invade. During EMT, epithelial cells are transformed into mesenchymal cells and vice versa. A number of markers that are characteristic of either epithelial or mesenchymal cell are listed (Xu et al., 2009).

3.1. Mesenchymal-to-epithelial transition and reprogramming

As already mentioned in chapter 2.5.1., several studies revealed that MET is involved in the early phase of the reprogramming process. The first indication that an early process of epithelisation is necessary for reprogramming came from a study by Mikkelsen *et al.* (2008). Using an integrative genomic analysis of fibroblasts undergoing reprogramming, they showed that EMT regulators, such as Tgfβ1, TgfβR2 and Snail, are reduced (Mikkelsen et al., 2008). Subsequently, two studies reported that inhibition of TGFβ-signalling enhances the reprogramming process of mouse fibroblasts (Ichida et al., 2009; Maherali and Hochedlinger, 2009). Maherali *et al.* (2009) demonstrated that the inhibition of TgfβR1 significantly increases the number of iPSC colonies, whereas activation of TGFβ-signalling by addition of exogenous Tgfβ1 and Tgfβ2 reduces the reprogramming efficiency. In addition, they showed that the TgfβR1-inhibitor is able to replace Sox2 or c-Myc in the reprogramming cocktail (Maherali and Hochedlinger, 2009). Similar results were obtained by Ichida *et al.* (2009), which showed that the use of a TgfβR1-inhibitor enhances reprogramming efficiency and replaces Sox2 in the reprogramming cocktail by inducing *Nanog* transcription. These studies implied that MET is required for reprogramming, which Samavarchi-Tehrani *et al.* and Li *et al.* formally demonstrated in 2010 (Li et al., 2010; Samavarchi-Tehrani et al., 2010). Li *et al.* (2010) demonstrated that the reprogramming factors activate an epithelial program and inhibit key mesenchymal gene expression. In particular, they showed the upregulation of cell-cell adhesion proteins and cytokeratins, as well as the reduced expression of mesenchymal markers, cell-matrix adhesion genes and transcriptional repressors linked with the mesenchymal state. In addition, OKSM stimulated a miRNA expression signature associated with MET, including downregulation of miR-155 and miR-10b, which have been linked to EMT (Ma et al., 2007; Kong et al., 2008), and upregulation of miR-205 and miR-429, which have been associated with MET (Gregory et al., 2008; Park et al., 2008). Looking at the contribution of each reprogramming factor, they showed that Sox2 and Oct4 suppress the EMT mediator Snail, c-Myc downregulates Tgfβ1 and TgfβR2, which are both involved in the induction of EMT, and Klf4 induces epithelial genes, including E-cadherin (Li et al., 2010). In the other study by Samavarchi-Tehrani *et al.* (2010), gene expression profiling revealed an induction of a large number of epithelial-associated genes, including E-cadherin, Occludin (Ocln) and Ep-Cam, during the first 5 days of the reprogramming. Consistent with the activation of epithelial-like markers in this early phase, mesenchymal regulators, including Snail, Slug, Zeb1 and Zeb2, were repressed. These findings support the hypothesis that initiation of reprogramming comprises a MET. Furthermore, they demonstrated that BMP signalling synergizes with OKMS to induce a miRNA expression pattern that promotes MET. Another study demonstrated that reprogramming is impaired in the absence of E-cadherin and that MET with an induced expression of E-cadherin is required (Redmer et al., 2011).

Combined, these studies demonstrate that MET is required for the generation of iPSCs and marks the early phase of the reprogramming process.

4. Poly(ADP-ribose) polymerase 1 (Parp1)/ADP-ribosyltransferase diphtheria toxin-like 1 (Artd1)

ADP-ribosyltransferase diphtheria toxin-like 1 (Artd1), also referred to as poly(ADP-ribose) polymerase 1 (Parp1), is an abundant and ubiquitous nuclear enzyme that is involved in a variety of nuclear processes. It is the founding member of the PARP family, which contains at least 17 distinct proteins (Amé et al., 2004; Schreiber et al., 2006; Hassa and Hottiger, 2008; Hottiger et al., 2010). Mammalian Artd1 is a 116-kDa protein consisting of well characterized structural and functional domains. The major functional units are an amino-terminal DNA-binding domain (DBD), a central automodification domain (AMD) and a carboxy-terminal catalytic domain (CD) (Amé et al., 2004; Schreiber et al., 2006). The DBD contains two zinc finger motifs that mediate binding to DNA, a zinc binding domain that mediates interdomain contacts, which are important for DNA-dependent enzyme activation, and a nuclear localization signal. The AMD comprises a breast cancer 1 protein C-terminus domain, which mediates protein-protein interactions, as well as several glutamate, aspartate and lysine residues, which act as putative acceptors for auto-(ADP-ribosylation). The CD contains a tryptophan-, glycine- and arginine-rich domain, an α-helical PARP regulatory domain that interacts with the substrate-binding site, and the highly conserved PARP signature motif, which binds NAD^+ (nicotinamide adenine dinucleotide) and defines the PARP family of proteins (D'Amours et al., 1999; Krishnakumar and Kraus, 2010). Together, these functional and structural domains make Artd1/Parp1 ideally suited to carry out a broad range of functions.

As a nuclear enzyme, Artd1 catalyses the covalent attachment of poly(ADP-ribose) (PAR) to itself (auto-PARylation) as well as to other substrates (PARylation), including histones, DNA repair proteins and transcription factors, using NAD^+ as a donor of ADP ribose units (Ogata et al., 1981; D'Amours et al., 1999; Kraus and Lis, 2003). Artd1 is involved in a broad variety of processes and functions via PARylation of target proteins or through binding to DNA and various proteins (Hassa and Hottiger, 2008). These processes include DNA repair, cell cycle regulation, cellular differentiation, and maintenance of genome stability and apoptosis (Kraus, 2008; Ji and Tulin, 2010; Krishnakumar and Kraus, 2010). Furthermore, Artd1 has been shown to be enriched at the promoters of actively transcribed genes, where it modulates the chromatin to regulate gene expression (Krishnakumar et al., 2008). It is also involved in transcription as a coregulator for DNA-binding transcription factors and as a regulator of DNA methylation (Kraus, 2008; Krishnakumar and Kraus, 2010). Despite all the roles that Artd1 plays in various biological processes, Artd1 knockout mice are viable and display only mild phenotypes (Wang et al., 1995). Nevertheless, Artd1-/- mice show some interesting phenotypes in

response to certain chemical agents, in some genetic backgrounds and under certain physiological conditions. For example Artd1-/- mice are susceptible to the spontaneous development of skin disease and are more sensitive to chemically induced genotoxic stress (Wang et al., 1995; de Murcia, J M et al., 1997; Wang et al., 1997). They also show resistance in various models of inflammation and increased tumor formation in chemically induced models of cancer (Morrison et al., 1997; Oliver et al., 1999; Tong et al., 2001; Ha, 2004). The mild phenotypes observed in Artd1 knockout mice may be due to compensatory effects mediated by other Artd family members. In this regard, Artd1 and Artd2 double knockout mice died at the early embryonic stages (Ménissier de Murcia, Josiane et al., 2003). The different roles of Artd1 in the regulation and maintenance of pluripotency will be discussed in the following chapter.

4.1. Artd1/Parp1 and stem cells

In the past years, researchers have shown that Artd1 plays different roles in the maintenance of pluripotency, in the differentiation of ESCs and in the generation of iPSCs.

In 1999, Masutani *et al.* generated Artd1-deficient (Artd1-/-) ESCs in order to study the biological functions of Artd1 in DNA damage responses. They demonstrated that Artd1-/- ESCs show an increased sensitivity to γ-irradiation and alkylating agents compared to parental ESCs. In addition, they could demonstrate *in vitro* culture that Artd1 deficiency is not lethal for ESCs and that their growth behaviour and morphology is not altered (Masutani et al., 1999). Subsequent studies investigated the differentiation potential of Artd1-/- ESCs *in vitro* and *in vivo*. Nozaki *et al.* (1999) injected Artd1-/- ESCs subcutaneously into nude mice to analyze the effect of *Artd1* disruption on tumourigenesis and cellular differentiation *in vivo*. Histological analysis of tumours derived from Artd1-/- ESCs showed the appearance of haemorrhagic areas as well as giant cells with single or multiple megalo-nuclei. Electron microscopic examination of the giant cells revealed micro-villi on the surface and secretion granules in the cytoplasma, both characteristically seen in trophoblasts in the normal placenta. In respect to all these features, the giant cells were diagnosed as syncytiotrophoblastic giant cells (STGCs). In contrast, none of the tumours derived from the wild-type (wt) cells or Artd1+/- clones displayed such haemorrhages or STGCs. However, apart from the presence of giant cells and haemorrhagic areas in Artd1-/- derived tumours, there was no difference in the ectodermal, mesodermal and endodermal tissue derivatives among wt, Artd1+/- and Artd1-/- tumours. These findings suggest that Artd1 is not essential for tumourigenesis of ESCs, but is involved in trophoblastic cell differentiation (Nozaki et al., 1999; Masutani et al., 2001). A follow-up study by Hemberger *et al.* (2003) demonstrated that trophoblast giant cells are not only present in tumours derived from Artd1-/- ESCs, but also in cultures of Artd1-/- ESCs, albeit at a low frequency (0.01%). In addition, the expression of spongiotrophoblast-specific marker genes provided evidence for the presence of other trophoblast subtypes in Artd1-/- tumours and ESCs (Hemberger et al., 2003). The induction of trophoblast

lineage in untreated and differentiated Artd1-/- ESCs is further supported by data from a microarray analysis of Artd1-/- ESCs and their wt counterparts (Ogino et al., 2007). This analysis revealed an up-regulation of certain genes in pluripotent and differentiating Artd1 -/- ESCs, which include genes regulating the differentiation into extraembryonic tissues, including trophoblast lineage, and genes representing well known markers of extraembryonic endoderm. In addition, genes involved in a variety of cellular processes, including proliferation, transcription, metabolism and cell structure, were down- or up-regulated in Artd1-/- ESCs (Ogino et al., 2007). Another study dissected the role of Artd1 in regulating transcription in differentiating ESCs (Gao et al., 2009). The data showed that Artd1 acts as a cofactor of Oct4 and Sox2 to modulate the expression of their target gene *Fgf4*. More precisely, Artd1 physically interacted with and PARylated Sox2, enabling the dissociation of excessive Sox2 from the *Fgf4* enhancer and inducing transcription of *Fgf4* (Gao et al., 2009). In Artd1-/- ESCs (undifferentiated and differentiating) the PARylation of Sox2 decreases, resulting in an increased association of Sox2 with *Fgf4* enhancer. This is accompanied by reduced expression of *Fgf4*. The reduced expression of *Fgf4* did not affect the proliferation and survival of undifferentiated Artd1-/- ESCs, however, the growth and survival of differentiating Artd1-/- ESCs was severely compromised. To summarize the results of the study, it was demonstrated that PARylation of Sox2 by Artd1 is important in modulating *Fgf4* expression during ESC differentiation (Gao et al., 2009). A subsequent study by Lai *et al.* (2012) also analyzed the role of Artd1 and Sox2 interaction in maintenance of ESC pluripotency and differentiation. They confirmed the finding of Gao *et al.* (2009) that Sox2 and Artd1 physically interact, however, they could not reproduce the PARylation of Sox2 by Artd1. In fact, their data showed that auto-PARylation of Artd1 enhances the Sox2-Artd1 interaction in ESCs, thereby controlling the binding of Sox2 to Sox2:Oct4 enhancers and preventing overexpression of Sox2:Oct4 target genes, such as *Fgf4* (Yuan et al., 1995), *Nanog* (Kuroda et al., 2005; Rodda et al., 2005), *Oct4* and *Sox2* itself (Catena et al., 2004; Chew et al., 2005). Therefore, auto-PARylated Artd1 together with Sox2 fine-tunes the level of Sox2:Oct4 target gene expression to maintain the pluripotent state of ESCs. In addition, they could demonstrate that inhibition of Sox2 activity by Sox2-PARylated Artd1 complexes plays an important role in the initiation of ESCs differentiation. In response to FGF/ERK signalling, which is an important signalling pathway for directing ESCs to exit self-renewal, Artd1 auto-PARylation enhanced Sox2-Artd1 interactions, which inhibited Sox2 activity. This finding was supported by the discovery that inhibition of the FGF/ERK pathway decreased Artd1 PARylation as well as Artd1-Sox2 interactions. Moreover, they revealed that Sox2-Artd1 interactions are present and also increase during iPSCs generation. Knockdown of Artd1 or inhibition of Artd1's enzymatic activity throughout the reprogramming process reduced the number of AP positive colonies significantly (Lai et al., 2012). Both the studies by Gao *et al.* (2009) and Lai *et al.* (2012) demonstrate the importance of the interplay between Artd1 and Sox2 in regulating the maintenance of pluripotency, differentia-

tion and even generation of iPSCs. Our own study supports the important role of Artd1 and Sox2 during reprogramming (Weber et al., 2013). We propose a unique mechanism in which Artd1 regulates reprogramming in concert with Sox2 and Fgf4. In agreement with Lai *et al.* (2012), we demonstrated that the reprogramming efficiency is significantly reduced in Artd1-/- MEFs and in wt MEFS reprogrammed in the presence of an inhibitor of PARP-mediated PARylation. In a subsequent step, we showed that the enzymatic activity of Artd1 is essential solely during the early phase and that the expression of Artd1 begins to regularly increase during the first days of the reprogramming process. Furthermore, our data revealed that Artd1 interacts with Sox2 and mediates its PARylation in the early phase of the process. The PARylation of Sox2 by Artd1 was shown to strengthen the binding of Sox2 to the *Fgf4* enhancer, thereby stimulating the expression of *Fgf4* throughout the reprogramming process. Interestingly, we could demonstrate that the expression of *Fgf4* is crucial for a successful and efficient reprogramming process (Weber et al., 2013). A recent study by Doege *et al.* (2012) revealed the important role of Artd1 together with Tet2 (ten-eleven translocation-2) in the regulation of epigenetic marks at pluripotency loci during reprogramming. Artd1 and Tet2 were recruited to the pluripotency loci of *Nanog* and *Esrrb* to promote an activated chromatin state that directs subsequent transcription of these pluripotency genes. Furthermore, Artd1 altered chromatin states in a way that the exogenous Oct4 binding to the pluripotency loci was facilitated (Doege et al., 2012).
Taken together, Artd1 is involved in numerous processes, including DNA repair, cellular differentiation and regulation of transcription. Artd1 also holds a modulating and regulating position in the acquisition and maintenance of pluripotency as well as in the differentiation of stem cells.

5. Fibroblast growth factors

Fibroblast growth factors (Fgfs) constitute a large family of structurally related polypeptides involved in a wide range of biological functions. They regulate development, cellular proliferation, growth and differentiation. The mammalian Fgf gene family consists of 22 members, including *Fgf1 – Fgf23*. *Fgf15,* however, has not been identified in humans and Fgf19 has not yet been found in mice and rats (Itoh and Ornitz, 2004, 2008). These Fgfs can be classified as intracrine, paracrine or endocrine Fgfs by their mechanism of action on target cells. The majority of Fgfs belong to the group of paracrine Fgfs and act as secreted proteins by binding to and activating cell surface tyrosine kinase FgfRs (fibroblast growth factor receptors), with heparan sulphate glycosaminoglycan as a cofactor. They modulate multiple developmental processes acting as local signalling molecules in an autocrine/paracrine manner (Itoh and Ornitz, 2008; Beenken and Mohammadi, 2009). Endocrine Fgfs function over long distances as endocrine hormones and mediate their biological responses in an FgfR-dependent way as well (Kharitonenkov, 2009; Itoh, 2010). In contrast, intracrine Fgfs neither activate FgfRs, nor are they secreted extracellularly. Their intracellular function is to interact with voltage gated sodium channels, where they mainly play a role in regulating the electrical excitability

of neurons and possibly of other cell types (Schoorlemmer and Goldfarb, 2002; Goldfarb et al., 2007; Laezza et al., 2009; Dover et al., 2010).

As already mentioned, endocrine and paracrine Fgfs carry out their diverse functions by binding and activating the Fgf receptor subfamily of cell-surface receptor tyrosine kinases. Four *FgfR* genes, *FgfR1, FgfR2, FgfR3 and FgfR4,* have been identified in humans and mice (Itoh and Ornitz, 2004). The Fgf receptors are transmembrane proteins consisting of three extracellular immunoglobulin (Ig)-like domains (D1-D3), an acidic region (a glutamate-, aspartate- and serine-rich sequence) between D1 and D2, a single transmembrane helix and a cytoplasmic domain with protein kinase activity (Coughlin et al., 1988; Lee et al., 1989; Dionne et al., 1990; Isacchi et al., 1990; Pasquale, 1990; Partanen et al., 1991; Johnson and Williams, 1993). As the different types of Fgfs have diverse effects on different target cells, the FGF-signalling system requires variation at the level of the FgfR. The FgfR diversity is ensured through the expression of splice variants of the *FgfR* genes. One splicing event results in the skipping of the exons encoding D1 and the D1-D2 linker, resulting in a two Ig-like domain form of the receptor. The binding properties of the “short” (two Ig-like domain) and the “long” (three Ig-like domain) FgfRs are similar (Johnson et al., 1991). A second alternative splicing event occurs in D3 of *FgfR1, FgfR2* and *FgfR3*. Two alternative exons (IIIb and IIIc) encode for the C-terminal half of D3 and are spliced to the exon IIIa, which codes for the first half of D3, in a mutually exclusive way. This alternative splicing event results in IIIb and IIIc receptor isoforms. The regulation of the alternative splicing in D3 occurs in a tissue- specific manner such that the IIIb isoform is predominantly expressed in epithelial tissues and the IIIc isoform is preferentially expressed in mesenchymal lineages (Avivi et al., 1993; Gilbert et al., 1993; Orr-Urtreger et al., 1993). D3, therefore, is an essential determinant of ligand-binding specificity (Zhang et al., 2006). As a result of these alternative splicing events occurring in D3, seven major FgfR proteins (FgfR1IIIb, FgfR1IIIc, FgfR2IIIb, FgfR2IIIc, FgfR3IIIb, FgfR3IIIc and FgfR4) with various ligand-binding specificity are generated from four *FgfR* genes.

Heparan sulphate glycosaminoglycan (HSGAG) acts as a cofactor for Fgfs when binding to and activating of FgfRs. It simultaneously binds Fgf and FgfR, thereby promoting and stabilizing protein-protein contacts between ligand and receptor. In addition, HSGAG protects Fgfs from degradation by endogenous proteases, acts as storage reservoir for ligands and retains the Fgfs at sites of functions, thus determining the radius of ligand diffusion (Allen, 2001; Häcker et al., 2005; Beenken and Mohammadi, 2009). The binding of ligand and HSGAG to the FgfR results in receptor dimerization, activation of intrinsic tyrosine kinase activity, and autophosphorylation at several tyrosine residues within the cytoplasmic domain of the receptor. Subsequently, adaptor proteins bind to the phosphorylated tyrosine residues on the receptor and activate the four key downstream transduction pathways: Jak/STAT (the Janus kinase/signal transducer and activator of transcription), PLCy (phosphoinositide phospholipase C), PI3K/AKT (phosphatidylinositol 3-kinase/ serine/threonine protein kinase B) and

MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase) pathways (Goldfarb, 2001; Dailey et al., 2005).

5.1. Fibroblast growth factor 4 (Fgf4) and stem cells

Fibroblast growth factor 4 (Fgf4) belongs to the paracrine Fgfs and preferentially binds to and activates the IIIc isoform of FgfR1-3 and FgfR4 (Ornitz et al., 1996; Zhang et al., 2006). Fgf4 plays a role in various biological processes, such as embryogenesis, tumourigenesis, and acquisition and maintenance of pluripotency in stem cells (reviewed in (Fernig et al., 2009). This chapter focuses on the functions of Fgf4 in murine stem cell biology.

In early murine embryonic development, *Fgf4* is the first member of the Fgf family to be expressed from the four-cell stage to the blastocyst (Rappolee et al., 1994). During the late blastocyst stage, the *Fgf4* expression becomes restricted to the epiblast cells of the ICM and, later in development as organogenesis is initiated, its expression can be detected in various tissues, including the myotome cells of mature somites, the branchial arches and the apical ectodermal ridge of the developing limb. However, the transcription of *Fgf4* is silenced in adults (Niswander and Martin, 1992). The deletion of *Fgf4* during embryonic development causes embryonic lethality within hours after implantation due to deficient ICM formation and maintenance. When Fgf4-/- embryos were cultured *in vitro*, the ICM showed an impaired proliferation unless Fgf4 was added to the culture medium causing rescue of growth and differentiation of the ICM (Feldman et al., 1995). In contrast, Fgf4-/- ESCs are able to survive, proliferate and maintain their pluripotent state *in vitro* independent of exogenous Fgf4 (Wilder et al., 1997). The same study, however, also showed that the differentiation of Fgf4-/- ESCs *in vitro* is severely compromised. The induction of differentiation in *Fgf4*-deficient ESCs resulted in limited growth and an impaired survival rate of the differentiating Fgf4-/- ESCs compared to their wt counterparts. However, the addition of Fgf4 to the culture medium was sufficient to increase the number of differentiated cells, in particular cells with many of the properties of extraembryonic endoderm (Wilder et al., 1997). A subsequent study by Kunath *et al.* (2007) demonstrated that Fgf4 is the major stimulus of ESCs activating the Ras-ERK signalling cascade. They showed that the absence of Fgf4 or inhibition of ERK activity did not disturb the expansion of undifferentiated ESCs. Instead, Fgf4-/- ESCs or ERK inhibited ESCs are unable to make the transition from pluripotency to differentiation. More precisely, these cells showed an impaired neuronal and mesodermal differentiation and also resisted differentiation in response to BMP4, while retaining the expression of key pluripotency factors (Kunath et al., 2007). Based on these findings, the authors proposed that autocrine FGF/ERK signalling is needed for naïve ESCs to exit self-renewal and initiate differentiation and that LIF and serum promote ESC self-renewal by overriding the pro-differentiation stimulus of FGF/ERK signalling. Interestingly, a subsequent study demonstrated that blockage of FGF/ERK signalling in combination with inhibition of GSK3 is sufficient to maintain the pluripotency of ESCs in absence of LIF (Ying et al.,

2008). As already mentioned, Fgf4 is essential for survival of the postimplantation embryo and plays important roles at various stages of embryonic development. Furthermore, it is expressed in undifferentiated ESCs and ECCs (Velcich et al., 1989; Schoorlemmer and Kruijer, 1991), where the transcription of *Fgf4* is controlled by Sox2 and Oct4 by binding on the distal *Fgf4* enhancer (Yuan et al., 1995). In addition, the expression of *Fgf4* is not limited to ESCs and ECCs, as it can be detected in iPSCs as well (Takahashi and Yamanaka, 2006). Interestingly, several studies showed that the expression of *Fgf4* starts in the early phase of the reprogramming process and constantly increases until reaching a steady-state level during the process (Araki et al., 2010; Buganim et al., 2012; Weber et al., 2013). In our study, we additionally showed that activation of the FgfR tyrosine kinases by Fgf4 is crucial for an efficient reprogramming process (Weber et al., 2013). In the follow-up study, we further analysed the role of Fgf4 during the generation of iPSCs. We could demonstrate that the exogenous addition of Fgf4 during d4-d8 of the reprogramming process increases the efficiency, whereas the inhibition of the FgfR tyrosine kinase activity by PD173074 (PD17) significantly reduces the efficiency. Furthermore, we showed that Fgf4 promotes reprogramming. It does this by regulating cell proliferation and favouring MET through parallel upregulation of epithelial gene expression and downregulation of mesenchymal genes (Weber et al., submitted).

Taken together, Fgf4 is involved in numerous processes such as embryonic development, cancer, and acquisition and maintenance of pluripotency in stem cells.

Aim of the study

Pluripotent stem cells are able to self-renew indefinitely and can differentiate into any of the numerous cell types of the body. Embryonic stem cells and induced pluripotent stem cells embody these features and therefore harbour a huge potential for clinical applications and improving the understanding of disease. The generation of iPSCs– unlike ESCs – does not have ethical implications and patient-derived iPSCs provide a valuable source for various clinical applications. The knowledge of the precise molecular mechanisms underlying the reprogramming of somatic cells to iPSCs is still limited. Enhancing the understanding of the reprogramming process may not only guide the design of improved reprogramming methods, which would lead to increased efficiency, but could also allow the generation of high-quality iPSCs.

The overall goal of this thesis is to shed light on the molecular mechanism underlying the reprogramming of somatic cells to pluripotent stem cells. Therefore, the following aims were addressed:

- Analyse the role of Artd1/Parp1 during the reprogramming process.
- Elucidate the functional role of the interaction between Artd1/Parp1 and Sox2 in the regulation of the *Fgf4* transcription.
- Determine the mechanism by which Fgf4 modulates and influences the reprogramming process.
- Define the time-frame during which the activation of the FGF-signalling pathway is crucial for an efficient reprogramming process.

B. Results

1. Artd1/Parp1 regulates reprogramming by transcriptional regulation of Fgf4 via Sox2 ADP-ribosylation

Authors

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Journal

Stem Cells, November 2013

Contribution

Designing experiments, performance and analysis of the following figures: Fig. 1A-B, Fig. 3A, Fig. 4, Suppl. Fig. 2, Suppl. Fig. 4. Designing experiments and partial performance (reprogramming of the cells): Fig. 1C-F, Fig. 2, Fig. 3B, Suppl. Fig. 1, Suppl. Fig. 3. PC supervised the project and wrote the manuscript together with FW.

Summary

The discovery made by Takahashi and Yamanaka that it is possible to reprogram somatic cells to iPSCs has opened new perspectives in the field of autologous cell therapy, regenerative medicine and drug screens. Since this momentous finding, many groups have worked on the refinement of reprogramming methods, however, the majority of them still struggle with a low efficiency, a long process length and/or the heterogeneity of the resulting iPSC population. Therefore, understanding the molecular mechanisms that underlie somatic cell reprogramming to pluripotency is crucial for the establishment of efficient and safe reprogramming techniques to generate high-quality iPSCs.

In our study we aimed to analyse the role of Artd1 and Sox2 in the regulation of the reprogramming process. We first addressed the question whether Artd1 influences reprogramming. For this purpose, we reprogrammed Artd1-/- fibroblasts and wt cells treated with a PARP-inhibitor according to Yamanaka's protocol (Takahashi et al., 2007a). The absence of Artd1 or the inhibition of its enzymatic activity reduced the reprogramming efficiency significantly. A subsequent time-point experiment revealed that the enzymatic activity is only essential during the early phase of the reprogramming process. In order to investigate the mechanism by which Artd1 modulates the reprogramming process, we analysed the possibility that Artd1 interacts with and PARylates Sox2 during the reprogramming process. Sox2 was previously shown to bind to and be modified by Artd1 in ESCs. Furthermore, it is one of the key factors regulating the pluripotency network in stem cells. We could show that Sox2 is targeted by ADP-ribosylation *in vitro* and that, during the first days of reprogramming, Artd1 interacts with and PARylates Sox2.

In a next step we demonstrated that the Sox2-Artd1 complex and the PARylation of Sox2 play a functional role during the reprogramming process. We showed that the expression of *Fgf4* in reprogramming wt fibroblasts constantly increases to day 6. In contrast, *Fgf4* expression was strongly reduced and delayed in Artd1-/- fibroblasts and in wt fibroblasts treated with PARP-inhibitor. Using chromatin immunoprecipitation, we revealed that Sox2 recruitment to the *Fgf4* enhancer is delayed in Artd1-/- cells as well as in PARP-inhibitor treated wt cells. These findings led to the hypothesis that ADP-ribosylation of Sox2 strengthens the binding of Sox2 to the *Fgf4* enhancer and thereby stimulates the transcription of *Fgf4*. We further analysed the interaction between Sox2 and Artd1 to see if its influence on *Fgf4* transcription causes the reduced reprogramming efficiency in Artd1-/- and PARP-inhibitor treated wt fibroblasts. To accomplish this, we added exogenous Fgf4 during the reprogramming of wt and Artd1-/- cells and could show that addition of Fgf4 is sufficient to restore the reprogramming efficiency of Artd1-/- cells to levels comparable to wt cells. Moreover, the addition of Fgf4 to PARP-inhibitor treated wt cells significantly enhanced the number of iPSC colonies compared to the number of colonies in wt cells solely treated with the PARP-inhibitor. In a subsequent experiment, we highlighted the importance of Sox2 and Artd1 in positively regulating *Fgf4* transcription. We showed that the inhibition of the FgfR tyrosine kinase activity by SU5402 reduces the reprogramming efficiency of wt fibroblasts by around 50%.
These findings demonstrate for the first time that Artd1-mediated PARylation of Sox2 is essential to positively modulate *Fgf4* transcription during the reprogramming process.

The paper (Weber, F.A., Bartolomei, G., Hottiger, M.O., and Cinelli, P. (2013). Artd1/Parp1 regulates reprogramming by transcriptional regulation of Fgf4 via Sox2 ADP-ribosylation. Stem Cells *31*, 2364-2373.) is reprinted and attached at the end of this thesis (Annex 1).

2. Fgf4 controls mesenchymal-to-epithelial transition during the early phases of reprogramming

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Journal

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Contribution

Designing experiments, performance and analysis of the following figures: Fig. 1A, Fig. 2, Fig. 3, Fig. 4, Fig. 5, Fig. 6, Suppl. Fig. 1. PC supervised the project and wrote the manuscript together with FW.

Summary

In our previous work, we showed that Artd1 interacts with and PARylates Sox2. PARylation of Sox2 favours its binding to the *Fgf4* enhancer, leading to an increase in *Fgf4* expression. Interestingly, we could further show that Fgf4 is a crucial factor for an efficient reprogramming process, as the inhibition of the FGF-signalling reduced the reprogramming efficiency.

In this study, we wished to identify the underlying mechanism by which Fgf4 modulates the reprogramming process. In a first attempt we aimed to confirm our previous data showing that inhibition of the FgfR tyrosine kinase activity reduces the reprogramming efficiency. We reprogrammed wt fibroblasts in the presence of either SU5402, as used in our previous publications, or PD173074 (PD17), an inhibitor of the FgfR tyrosine kinases with a higher affinity for the FgfR. The number of iPSCs colonies obtained through reprogramming was reduced by 50% using SU5402 and by 70% using PD17. In order to investigate the molecular mechanism underlying the reduction of reprogramming efficiency by PD17, we performed a microarray analysis of retroviral transduced MEFs at day 4 in the presence or absence of PD17. This analysis showed that the inhibition of the FGF-signalling pathway affects genes related to EMT/MET.

To further confirm the effect of the FGF pathway on reprogramming, we decided to use mouse secondary MEFs (2nd MEFs) carrying a single doxycycline-inducible polycystronic cassette harbouring all four reprogramming factors (Carey et al., 2009). Reprogramming of 2nd MEFs in the presence of PD17 or neutralizing anti-FGF4 antibodies resulted in a decreased reprogramming efficiency. In contrast, addition of exogenous FGF4 during the reprogramming process of 2nd MEFs significantly enhanced the number of iPSC colonies compared to the number in untreated 2nd MEFs. In a subsequent step, we were interested in which phase of the reprogramming process the FGF-signalling is most important. Therefore, we segmented the reprogramming process into three phases (d0-d4, d4-d8 and d8-d12). We could demonstrate that activation of the FGF pathway by FGF4 during d4 – d8 is most crucial for an efficient reprogramming process and that complementation of the medium with additional FGF4 in this phase significantly improves the reprogramming efficiency.

As demonstrated in our transcriptomic analysis, the addition of PD17 during the reprogramming process affects a number of genes involved in EMT/MET. It has already been shown that the induction of MET is an essential event during the first days of the reprogramming process. Therefore, we analysed what effect the addition of PD17 and FGF4 during the reprogramming process has on the MET-related genes. We could show that addition of FGF4 favours MET by upregulating the expression of epithelial genes and downregulating the expression of mesenchymal genes. In contrast, presence of PD17 during the reprogramming process caused an upregulation of the mesenchymal-related genes Zeb2, Snail and fibronectin. Zeb2 and Snail are known to maintain the mesenchymal phenotype by directly repressing epithelial gene expression. The activation of Snail occurs downstream of TGFβ-

signalling, a well-known EMT inducer. We therefore wondered whether the inhibition of the TGFβ pathway and the subsequent suppression of EMT could rescue the observed negative effect of PD17 during reprogramming of 2nd MEFs. Interestingly, the inhibition of the TGFβ pathway in PD17 treated cells between d0-d4 and d8-d12 increased the reprogramming efficiency to a level comparable to untreated 2nd MEFs. However, the inhibition of the TGFβ pathway between d4-d8 was not sufficient to rescue the PD17 phenotype, highlighting the importance of Fgf4 to drive MET in the central phase of the reprogramming process.
Combined, our work was the first to identify Fgf4 as an important factor for promoting reprogramming by favouring MET.

The manuscript (Weber, F.A.; Graf, U.; Evantal, A.; Biran, A.; Okoniewski, M.J.; Meshorer, E.; Cinelli, P. Fgf4 controls mesenchymal-to-epithelial transition during the early phases of reprogramming) is attached at the end of this thesis (Annex 2).

C. Discussion and Outlook

The discovery by Takahashi and Yamanaka that MEFs can be reprogrammed to iPSCs has been recognized as a fundamental breakthrough in biology and medicine (Takahashi and Yamanaka, 2006). Over the past years, the reprogramming techniques have been improved with regard to vector delivery methods, cell sources or reprogramming factor combinations (Hussein and Nagy, 2012; Miyazaki et al., 2012). Despite all the advances in the optimization of reprogramming techniques, knowledge of the underlying mechanisms and how the four reprogramming factors Oct4, Sox2, Klf4 and c-Myc drive the process of reprogramming is still limited. A better mechanistic understanding of the reprogramming process, including the role of the four reprogramming factors, would help improve reprogramming methods and could allow the generation of high-quality iPSCs.

In our work, the first aim was to understand the functional consequence of the interplay between Sox2 and Artd1 during the early phase of the reprogramming process. We could demonstrate that Artd1 PARylates and interacts with Sox2, which has a modulating function on the Sox2-driven *Fgf4* expression during the reprogramming process. We could further show that Fgf4 plays an important role during the reprogramming process. More precisely, Fgf4 positively supports MET, which is a hallmark of the early phase of reprogramming.

1. Artd1/Parp1 regulates reprogramming by transcriptional regulation of Fgf4 via Sox2 ADP-ribosylation

The four transcription factors Oct4, Sox2, Klf4 and c-Myc were originally used to reprogram fibroblasts to iPSCs (Takahashi and Yamanaka, 2006). Although various reprogramming factor combinations have been established over the last years, the four Yamanaka factors still represent the most commonly used components of the reprogramming cocktail. However, knowledge of how the Yamanaka factors directly drive reprogramming is still limited. In ESCs, Oct4 and Sox2 are key players of the transcriptional network regulating maintenance of pluripotency (Nichols et al., 1998; Avilion et al., 2003). Within this network, they cooperatively regulate their own expression as well as the transcription of additional target genes, such as *Nanog*, *Fgf4*, *Lefty1* and *Fbx15*, by binding to their Oct4:Sox2 enhancer elements (Dailey et al., 1994; Yuan et al., 1995; Tokuzawa et al., 2003; Kuroda et al., 2005; Okumura-Nakanishi et al., 2005; Nakatake et al., 2006). Therefore, the regulation of the two core factors, Sox2 and Oct4, is an important determinant of pluripotency. One enzyme that has been demonstrated to post-translationally regulate Sox2 is Artd1. Artd1 is a chromatin associated factor that catalyses the covalent attachment of PAR to itself and to other nuclear acceptor proteins (D'Amours et al., 1999). In ESCs, Artd1 was previously shown to be necessary for proper differentiation (Hemberger et al., 2003). Even though Artd1 deficiency does not affect the growth of ESCs, its

absence compromises cell survival and growth when ESCs are induced to differentiate (Hemberger et al., 2003; Gao et al., 2009). In this work, we aimed at understanding the function of Artd1 and Sox2 during the first phase of reprogramming. We first tested the reprogramming capacity of Artd1 knockout fibroblasts and found that the absence of Artd1 strongly reduces the number of iPSC colonies. We could also identify that the critical days during which the enzymatic activity of Artd1 is necessary are the first 2-4 days after transduction with the reprogramming factors. This is in agreement with the increased expression of Artd1 starting at day 2 after reprogramming and the concomitant increase of PARylation. We further observed a strong delay in *Fgf4* expression upon the initiation of reprogramming in Artd1 -/- fibroblasts or when wt fibroblasts were treated with ABT-888, an inhibitor of poly-ADP-ribosyltransferases (Figure 7). *Fgf4* expression occurs much earlier than the activation of transcription of other typical pluripotency markers such as Nanog, SSEA1 or Oct4. This observation is interesting because two previous studies in ESCs showed that Artd1 and Sox2 are involved in the regulation of *Fgf4* expression (Gao et al., 2009; Lai et al., 2012). A study by Lai *et al.* (2012) showed that the binding of Sox2 to the enhancers of its target genes, including *Fgf4*, is regulated by Artd1. More precisely, they demonstrated that Artd1 auto-PARylation enhances Sox2-Artd1 interactions. The consequence of this interaction was the inhibition of the binding of Sox2 to the Oct4:Sox2 enhancers. In agreement with this finding, the inhibition of the enzymatic activity of Artd1 resulted in an enhanced Sox2 binding to Oct4:Sox2 elements and subsequently in overexpression of Oct4:Sox2 target genes (Lai et al., 2012). An alternative model suggests that during differentiation of ESCs Artd1 directly interacts with and PARylates Sox2, which leads to the dissociation and degradation of Sox2 from the *Fgf4* enhancer. This releases Sox2 inhibition and induces *Fgf4* gene transcription (Gao et al., 2009). In the absence of activated Artd1, Sox2 cannot be PARylated, which increases its interaction with the *Fgf4* enhancer and leads to a reduction in Fgf4 levels (Gao et al., 2009). In conclusion, both the studies showed that Artd1 is responsible for regulating the Sox2-driven expression levels of the Oct4:Sox2 target genes. Based on these two studies and our finding that *Fgf4* expression is delayed in Artd1-/- and ABT-888 treated wt cells, we decided to analyse the capacity of Artd1 to modify Sox2 and a possible influence of these two proteins on the *Fgf4* expression. Our data demonstrate that Artd1 is able to PARylate Sox2 *in vitro* and strongly suggest that Artd1 mediates ADP-ribosylation of Sox2 *in vivo* in fibroblasts starting from day 2 during reprogramming. Additionally, Artd1 interacts with Sox2 during the reprogramming process. To further examine whether the Artd1-Sox2 interaction and the PARylation of Sox2 have an influence on *Fgf4* expression, we performed a chromatin immunoprecipitation against Sox2 and analysed the binding of Sox2 to the *Fgf4* enhancer. Our data show that Sox2 recruitment to the *Fgf4* enhancer is reduced and delayed in Artd1-/- and ABT-888-treated cells compared to that in wt cells. This is in agreement with the delayed and reduced *Fgf4* expression in Artd1-/- fibroblasts and wt cells treated with ABT-888. These findings suggest that

Artd1 mediated PARylation of Sox2 is necessary for the efficient binding of Sox2 to the *Fgf4* enhancer and for inducing *Fgf4* expression (Figure 7), which in turn is responsible for initiating the events leading to the formation of iPSCs.

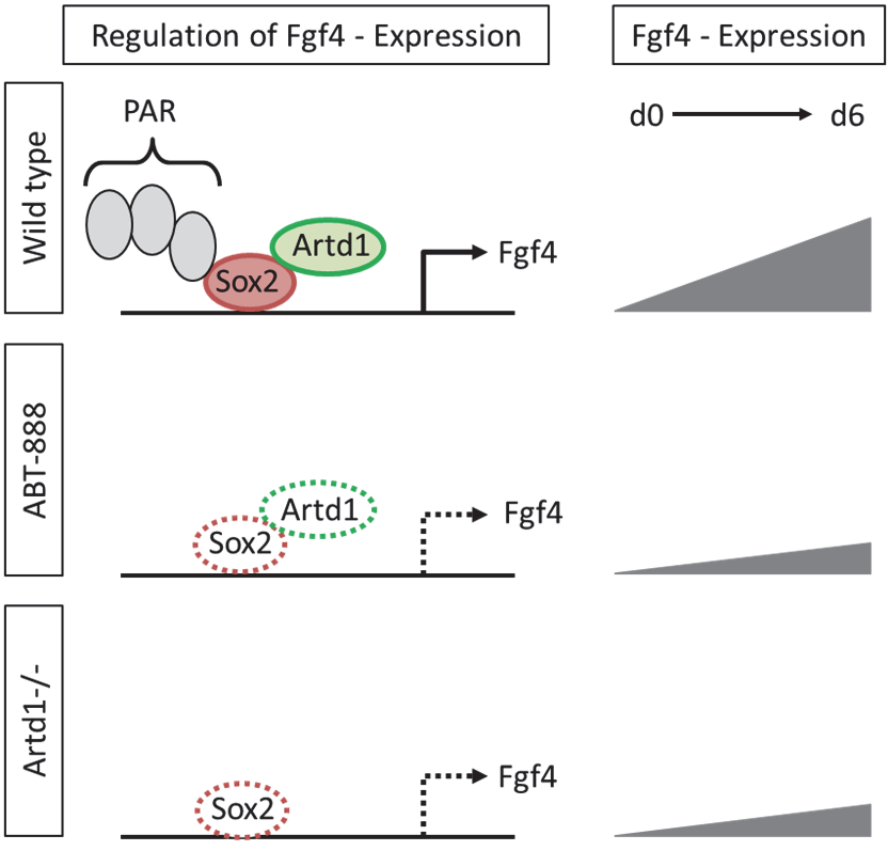

Fig. 7: Regulation of Fgf4 expression in the early days of the reprogramming process

During the reprogramming process, Sox2 is recruited to the *Fgf4* enhancer. In wild-type cells, ADP-ribosylation of Sox2 strengthens the binding of Sox2 to the *Fgf4* enhancer and thereby stimulates the transcription of the *Fgf4* gene. In ABT-888 treated wild-type cells and in Artd1-deficient cells, the recruitment to the *Fgf4* enhancer is reduced and delayed. Therefore, the increase in the *Fgf4* expression over the first six days of the reprogramming process is reduced.

The importance of Fgf4 during the first phase of the reprogramming process is reinforced by the fact that Artd1-/- fibroblasts, which show a strongly reduced activation of *Fgf4* upon reprogramming initiation, display a massive reduction in the number of iPSC colonies. The simple addition of Fgf4 during this time is sufficient to restore the reprogramming efficiency to levels comparable to those seen in wt cells, indicating that Fgf4 is the only factor regulated by Artd1 during the early phase of reprogramming. The importance of Fgf4 during the early phase is also corroborated by the fact that treating wt cells with an inhibitor of FgfR tyrosine kinases, which are normally activated upon the binding of Fgf4, reduces the reprogramming efficiency significantly. In summary, our data highlight the importance of Artd1 and Sox2 in regulating *Fgf4* expression during the first days of the reprogramming

process. Our findings illustrate one role of Artd1 during the reprogramming process, while studies conducted by two other groups have shown additional functions of Artd1 during reprogramming (Doege et al., 2012; Chiou et al., 2013). However, little is known about the role of Artd1 in iPSCs. Although the reprogramming efficiency of Artd1-/- cells was reduced, we succeeded in generating Artd1-/- iPSCs. From various studies, we know that the growth behaviour and morphology of *Artd1*-deficient ESCs is not altered, but its absence compromises cell survival and growth when ESCs are induced to differentiate (Masutani et al., 1999; Gao et al., 2009). Additional studies demonstrated that tumours derived from Artd1-/- ESCs showed the appearance of trophoblast giant cells. However, apart from the presence of these giant cells, there was no difference in the ectodermal, mesodermal and endodermal tissue derivatives among wt and Artd1-/- tumours (Nozaki et al., 1999; Masutani et al., 2001). The growth behaviour and morphology of our Artd1-/- iPSCs was not affected compared to wt iPSCs and they expressed various markers associated with pluripotency. Furthermore, the Artd1-/- iPSCs were able to differentiate *in vitro* toward smooth muscles and neurons (Weber et al., 2013). It would be interesting to test the differentiation ability of Artd1-/- iPSCs *in vivo* and check for the appearance of trophoblast giant cells, to see if Artd1-/- iPSCs show differentiation properties similar to *Artd1*-deficient ESCs.

2. Fgf4 controls mesenchymal-to-epithelial transition during the early phases of reprogramming

In our previous study, we demonstrated that *Fgf4* expression during the early days of the reprogramming process is regulated by Artd1 and Sox2. Furthermore, we could show that inhibition of the FgfR tyrosine kinase activity significantly reduces reprogramming efficiency (Weber et al., 2013). These findings served as a starting point for our follow-up project. Several studies already demonstrated that the expression of *Fgf4* begins in the early phase of the reprogramming process and constantly increases until reaching a steady-state level at the end of the process (Araki et al., 2010; Buganim et al., 2012; Weber et al., 2013). In addition, the expression of *Fgf4* can be detected in iPSCs (Takahashi and Yamanaka, 2006). However, all of these studies did not analyse the role that Fgf4 possibly plays during the reprogramming process. In ESCs, the major function of Fgf4 is to regulate the selection between the alternative fates of self-renewal and lineage commitment during continuous proliferation. More precisely, the absence of Fgf4 does not compromise the expansion of undifferentiated ESCs. Instead, Fgf4-/- ESCs are unable to make the transition from pluripotency to differentiation (Kunath et al., 2007). Kunath *et al.* (2007) further showed that Fgf4 mediated activation of the FGF/ERK signalling cascade is needed for ESCs to exit self-renewal and initiate differentiation. As ESCs express *Fgf4* and are therefore continuously subjected to the differentiation stimulus triggered by Fgf4, serum and LIF are required to override the pro-differentiation stimulus of FGF/ERK signalling

(Kunath et al., 2007). In our work, we were interested in analysing the role of Fgf4 during the generation of iPSCs. We therefore performed a microarray analysis to compare global gene expression changes occurring at day 4 upon retrovirus-mediated transduction of MEFs in the presence or absence of PD17. The small-chemical inhibitor PD17 blocks the FgfR tyrosine kinase activity and therefore inhibits the pathways normally activated by Fgf4. This analysis indicated that inhibition of the FGF pathway during reprogramming affects genes related to cell cycle progression and EMT/MET (Weber et al., submitted manuscript). To verify the results of the microarray analysis, we switched the reprogramming system from retrovirus-mediated reprogramming to the secondary reprogramming system. Secondary systems usually use somatic cells carrying doxycycline-inducible reprogramming factors to generate secondary iPSCs. These somatic cells containing doxycycline-inducible reprogramming factors are generated from primary iPSCs, which were differentiated to a somatic cell state by *in vivo* embryonic development, either through chimera formation or in transgenic offspring. This system allows the reprogramming of genetically homogenous somatic cell populations while avoiding the genetic heterogeneity of primary infections. Furthermore, it enables temporally-controlled induction of expression of the reprogramming factors as well as higher reprogramming efficiency. Because of this, the majority of the studies investigating the mechanisms underlying the reprogramming process have made use of the secondary system (Nagy, 2013). We used 2nd MEFs carrying a single doxycycline-inducible polycystronic cassette containing all four reprogramming factors (Carey et al., 2009). This enabled us to make use of all the aforementioned advantages of secondary systems in the examination of the role of Fgf4 during the reprogramming process. In a first step, we tested the response of 2nd MEFs to inhibition (by PD17) or activation (by human FGF4) of the FGF pathway. The obtained results demonstrated that blocking of FGF-signalling reduced the reprogramming efficiency, whereas activation of the FGF pathways enhanced the reprogramming efficiency compared to wt levels. To further test the importance of Fgf4, we reprogrammed 2nd MEFs in the presence of neutralizing anti-FGF4 antibodies. The reprogramming efficiency under this condition was also significantly reduced compared to wt conditions (Weber et al., submitted manuscript). On one hand, these experiments demonstrate that inhibition or activation of the FGF-signalling have a significant effect on the reprogramming efficiency. On the other hand, they show that specifically Fgf4 is crucial for an efficient reprogramming process. Nevertheless, these results do not reveal whether FGF-signalling is important during the entire reprogramming process or only during a specific time frame. A study by Samavarchi-Tehrani *et al.* (2010) showed that the reprogramming process can be subdivided into three phases: initiation, maturation and stabilization. The hallmarks of the initiation phase are the loss of gene expression associated with somatic cells, an increased proliferation rate, morphological changes and the initiation of MET. The subsequent phase, the maturation phase, is marked by the activation of a subset of pluripotency associated genes. In

the last phase, the stabilization phase, the cells become independent of the transgenes and display typical characteristics and markers of pluripotency (Li et al., 2010; Samavarchi-Tehrani et al., 2010; Buganim et al., 2012; Polo et al., 2012; Buganim et al., 2013; Sancho-Martinez and Izpisua Belmonte, Juan Carlos, 2013; David and Polo, 2014). In order to discover during which period of the reprogramming process FGF-signalling is important, we segmented the reprogramming process into three phases: d0 – d4, d4 – d8 and d8 – d12. We could demonstrate that the inhibition of the FGF pathways, either by PD17 or neutralizing anti-FGF4 antibodies, has a negative effect on the reprogramming efficiency independent from the phase. However, the most significant reduction in the efficiency occurred when the FGF-signalling was inhibited during d4 – d8 (Fig. 8A/B). We also wondered whether the positive effect of Fgf4 on the reprogramming efficiency is phase dependent. Interestingly, the addition of FGF4 during d0 – d4 had no effect, whereas the addition of FGF4 during d4 – d8 caused a significant increase in the number of iPSC colonies. This increase was comparable to the increased number of iPSC colonies seen when 2nd MEFs were treated with FGF4 during the entire process. Finally, the presence of FGF4 during the last phase (d8 – d12) had a negative impact on the reprogramming efficiency (Fig. 8C). These results clearly indicate that activation of the FGF-signalling by Fgf4 is most important during d4 – d8 of the reprogramming process. From a microarray analysis, we knew that the inhibition of FGF-signalling mainly affects genes related to cell cycle progression and MET/EMT (Weber et al., submitted manuscript). A previous study by Hanna *et al.* (2009) revealed that an increase in cellular proliferation is needed for the acquisition of stochastic epigenetic changes during reprogramming (Hanna et al., 2009). Furthermore, several studies showed that acceleration of proliferation is an important early event of reprogramming (Mikkelsen et al., 2008; Hong et al., 2009; Marión et al., 2009; Utikal et al., 2009). Interestingly, it was already demonstrated that Fgf4 enhances proliferation of ESCs (Kook et al., 2013). Taking all these studies into account, we wondered whether the positive effect of Fgf4 on the reprogramming process could be caused by increasing the cellular proliferation. Our data show that cell cycle inhibitory genes are downregulated in FGF4 treated cells and upregulated in the 2nd MEFs treated with PD17 (Weber et al., submitted manuscript). Based on these results it is reasonable to conclude that activation of FGF-signalling has a positive effect on the cell cycle during reprogramming. Further experiments would be needed to analyse the influence of Fgf4 on the cell-division rate during reprogramming more precisely. Besides the influence of FGF-signalling on cell cycle progression, our microarray analysis demonstrated that inhibition of the FGF pathway also affects MET/EMT-associated genes. During development, EMT is essential for the proper formation of the body plan and the differentiation of cells into many tissues and organs. During this process, epithelia convert to mesenchyme through multiple rounds of EMT accompanied by the reversible process of MET (Acloque et al., 2009). In adult tissues, the EMT program is reactivated during specific processes like wound healing, organ fibrosis and tumour progres-

sion (Zeisberg and Neilson, 2009). EMT/MET play pivotal roles not only during organ development, but also in cancer metastasis by providing cells with migratory and invasive properties (Thiery et al., 2009). Of note, during reprogramming, fibroblasts gradually lose their differentiated identity and acquire ESC gene expression patterns and growth behaviour associated with pluripotency. Morphologically, this conversion is linked to a transition from a single layer of adherent cells to a multilayer of epithelial cells; a process that strongly resembles MET. Recent studies indeed confirm that MET is a crucial process during the early reprogramming of murine embryonic fibroblasts into iPSCs. One of these studies by Samavarchi-Tehrani *et al.* (2010) demonstrated an activation of epithelial markers and a repression of mesenchymal regulators during the first days of the reprogramming process. This study, in combination with additional studies (Mikkelsen et al., 2008; Stadtfeld et al., 2008; Sridharan et al., 2009; Li et al., 2010), shows that MET is required for the generation of iPSCs and marks the early phase of the reprogramming process. Various members of the Fgf family have already been shown to influence the process of MET. For example, Ramos *et al.* (2010) revealed that FGF1 is able to revert Tgfβ-induced EMT in human and rat alveolar epithelial-like cell lines (Ramos et al., 2010). A study by Jiao *et al.* (2013) is of even more interest. It showed that mesenchymal and Tgfβ family genes were downregulated in cells treated with Fgf2 during the reprogramming process. They concluded that addition of Fgf2 during reprogramming facilitates the induction of MET (Jiao et al., 2013). We analysed the gene expression profiles of MET-associated genes in the presence of PD17 or FGF4 and revealed that the expression levels of epithelial genes were upregulated in FGF4 treated 2nd MEFs and unchanged in the presence of PD17. The presence of FGF4 also downregulated the expression levels of several mesenchymal-associated genes, whereas addition of PD17 caused an upregulation of the mesenchymal-related genes Zeb2, Snail and fibronectin (Weber et al., submitted manuscript). Taken together, this data indicate that activation of the FGF pathway supports MET during the early days of the reprogramming process. Based on this discovery, we wondered if a crosstalk of FGF-signalling with other pathways is also involved. One of the signalling pathways closely related to EMT/MET is the TGFβ-pathway. Various studies have already shown that TGFβ-signalling plays an important role in EMT by activating the well-known EMT regulator Snail (Peinado, 2003; Cho et al., 2007; Thiery et al., 2009). Two interesting back-to-back studies reported that inhibition of TGFβ-signalling positively influences reprogramming efficiency (Ichida et al., 2009; Maherali and Hochedlinger, 2009). As mentioned above, our study showed that inhibition of FGF-signalling by PD17 reduces the reprogramming efficiency significantly. Assuming that Tgfβ-induced EMT counteracts Fgf4-induced MET during the reprogramming process, we wondered whether the blocking of TGFβ-signalling could rescue the observed negative effect of PD17. To test this, 2nd MEFs were reprogrammed in the presence of both PD17 and A83-01 (an inhibitor of the type 1 Tgfβ receptor) between d0 – d4, d4 – d8 or d8 – d12. The addition of both the inhibitors between d0 – d4 and d8 – d12

increased the reprogramming efficiency to a level comparable to the wt level. In contrast, the inhibition of the TGFβ-pathway between d4 – d8 was not sufficient to rescue the reduced reprogramming efficiency in PD17 treated cells (Fig. 8D) (Weber et al., submitted manuscript).

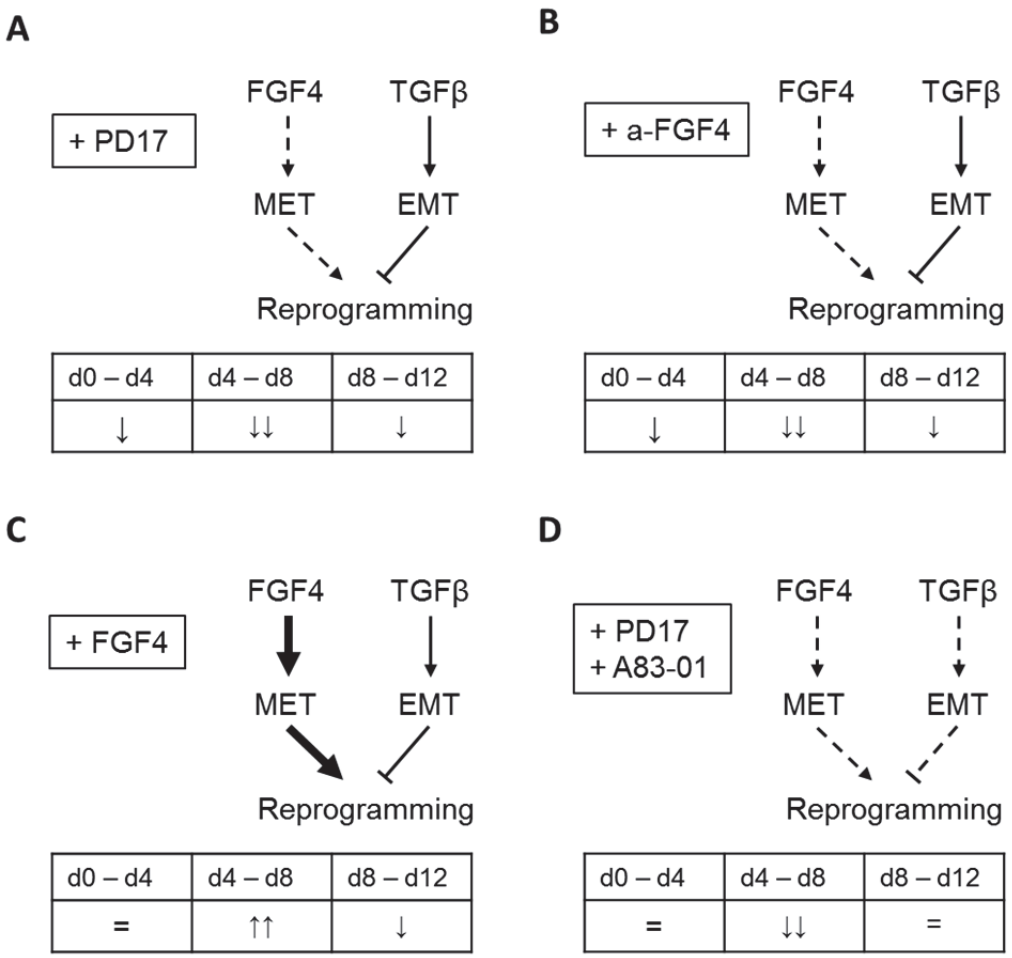

Fig. 8: Modulation of EMT/MET by FGF- and TGFβ influences the reprogramming process

The reprogramming process requires activation of MET in the early phases as well as suppression of EMT for a successful and efficient reprogramming process. FGF-signalling is involved in the regulation of MET, whereas TGFβ-signalling promotes EMT. **A)** Inhibition of the FgfR tyrosine kinases activity by PD17 is adverse during all three phases of the reprogramming process, but the strongest impact can be observed during d4-d8. **B)** Neutralization of Fgf4 by the anti-FGF4 antibody reduces the reprogramming efficiency throughout all phases, but most significantly between d4-d8. **C)** FGF4 is able to increase the reprogramming efficiency by promoting MET, if added between d4-d8. **D)** Inhibition of FGF-signalling by PD17 and the resulting negative effect on MET can be compensated in the early and late phase by blocking TGFβ-signalling with A83-01. The addition of A83-01 is not sufficient to rescue the PD17 phenotype in the central phase (d4-d8) (from Weber et al., submitted manuscript).

These results demonstrate that the blocking of FGF-signalling by PD17 and the resulting negative effect on MET can be compensated in the early and late phase by inhibiting TGFβ-signalling. Between d4 – d8, blocking of TGFβ-signalling cannot compensate for the inhibited FGF-signalling. These results highlight the importance of Fgf4 to activate the FGF pathway and drive MET between d4 – d8 of the reprogramming process.

3. Outlook – Future perspectives and concluding remarks

Pluripotent stem cells are able to self-renew for an indefinite time period, while maintaining the capability to differentiate into any cell types of the body. iPSCs embody these features and therefore offer an invaluable source of patient-derived iPSCs for disease modelling, drug screening, toxicology tests and regenerative medicine. Despite all the advances in the field of somatic cell reprogramming, the knowledge of the precise molecular mechanisms that underlie the reprogramming process is still limited. However, a better understanding of reprogramming mechanisms may lead to improved reprogramming techniques and to the generation of high-quality iPSCs.

In the first of our studies reported in this Thesis, we identified Artd1 as a regulator for the expression of *Fgf4*, which is an important factor during the reprogramming process. Considering the fact that Artd1 is involved in a variety of processes, including transcription regulation and DNA methylation, it is reasonable to assume that Artd1 may be involved in additional processes during the reprogramming process. This assumption is supported by two studies showing that Artd1 promotes reprogramming in the absence of c-Myc (Chiou et al., 2013) and is involved in the regulation of epigenetic marks at pluripotency loci during reprogramming (Doege et al., 2012). To investigate how broad the involvement of Artd1 is in the mechanisms of the reprogramming process, an analysis investigating changes of the transcriptome and proteome during the reprogramming of Artd1-/- cells would offer a good starting point. In this case, the use of a secondary system would be preferable, due to the possibility of studying reprogramming in homogenous populations of cells. Apart from the enormous potential to study the role of Artd1 during the reprogramming process, our project could also serve as a basis for analysing the behaviour of Artd1-/- iPSCs. It is known that absence of Artd1 in ESCs compromises their *in vitro* differentiation potential and that tumours derived from Artd1-/- ESCs contain trophoblast giant cells. Therefore, a comparative analysis of Artd1-/- ESCs and iPSCs would offer valuable input on the differences and/or similarities between these two types of *Artd1*-deficient pluripotent stem cells.

In our second project, we demonstrated that Fgf4 plays an important role during the reprogramming process, namely in the early phase where it supports MET. As it is known that binding of Fgf4 to the FgfRs results in the activation of four downstream transduction pathways, it would be of great interest to analyse which particular pathway(s) is/are involved in the initiation of MET. This could be achieved by inhibiting specific downstream pathways of Fgf4 during the reprogramming process and carefully analysing the occurrence or absence of MET. Furthermore, we could show that TGFβ-signalling counteracts the initiation of MET by promoting EMT. In this context, it would be interesting to examine which factor(s) mediate the crosstalk between FGF- and TGFβ-signalling. These approaches would provide further insights into the mechanisms of the reprogramming process. To better understand the pluripotency of iPSCs, it would perhaps be interesting to analyse the function of

Fgf4 in iPSCs. It is known that FGF/ERK-signalling activated by Fgf4 is needed for ESCs to exit self-renewal and initiate differentiation. To my knowledge, the question of whether Fgf4 plays the same role in iPSCs is still unanswered. An answer to this question would offer insight into the maintenance of the pluripotent state of iPSCs.

Taken together, all the knowledge gained from both our study and others about the mechanisms underlying the reprogramming process should pave the way for the use of iPSCs for medical applications.

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Annex 1

Artd1/Parp1 Regulates Reprogramming by Transcriptional Regulation of Fgf4 Via Sox2 ADP-Ribosylation

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Artd1/Parp1 Regulates Reprogramming by Transcriptional Regulation of Fgf4 Via Sox2 ADP-Ribosylation

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Key Words. Cell biology • Induced pluripotent stem cells • Artd1/Parp1 • Pluripotent stem cells • Reprogramming • Fgf4

ABSTRACT

The recently established reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) by Takahashi and Yamanaka represents a valuable tool for future therapeutic applications. To date, the mechanisms underlying this process are still largely unknown. In particular, the mechanisms how the Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc) directly drive reprogramming and which additional components are involved are still not yet understood. In this study, we aimed at analyzing the role of ADP-ribosyltransferase diphtheria toxin-like one (Artd1; formerly called poly(ADP-ribose) polymerase 1 [Parp1]) during reprogramming. We found that poly(ADP-ribosylation) (PARylation) of the reprogramming factor Sox2 by Artd1 plays an important role during the first days upon transduction with the reprogramming factors. A process that happens before Artd1 in conjunction with 10–11 translocation-2 (Tet2) mediates the histone modifications necessary for the establishment of an activated chromatin state at pluripotency loci (e.g., Nanog and Essrb) [Nature 2012;488:652–655]. Wild-type (WT) fibroblasts treated with an Artd1 inhibitor as well as fibroblasts deficient for Artd1 (Artd1−/−) show strongly decreased reprogramming capacity. Our data indicate that Artd1-mediated PARylation of Sox2 favors its binding to the fibroblast growth factor 4 (*Fgf4*) enhancer, thereby activating Fgf4 expression. The importance of Fgf4 during the first 4 days upon initiation of reprogramming was also highlighted by the observation that exogenous addition of Fgf4 was sufficient to restore the reprogramming capacity of Artd1−/− fibroblast to WT levels. In conclusion, our data clearly show that the interaction between Artd1 and Sox2 is crucial for the first steps of the reprogramming process and that early expression of Fgf4 (day 2 to day 4) is an essential component for the successful generation of iPSCs. STEM CELLS *2013;31:2364–2373*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The recent discovery of Takahashi and Yamanaka that it is possible to establish pluripotent stem cells, so called induced pluripotent stem cells (iPSCs), by reprogramming differentiated somatic cells [1] has opened new perspectives in the field of regenerative medicine. Therefore, many groups have worked on the refinement of reprogramming in order to optimize this technology for the use of iPSCs in clinical applications (reviewed in [2]). Nevertheless, the molecular mechanisms underlying the process of reprogramming are still largely unknown. An increased knowledge on how this process is driven and on the underlying mechanisms would lead to improved, more efficient reprogramming techniques.

Originally, reprogramming of mouse and human fibroblasts to iPSCs was performed by the retroviral-mediated introduction of the four transcription factors, Oct 4, Sox2, Klf4, and c-Myc (the so called Yamanaka factors [1]). How these factors directly drive the process of reprogramming and which additional components are involved still needs to be carefully analyzed. One of the Yamanaka factors, the transcription factor Sox2 (sex determining region Y-box 2), is a main player in maintaining pluripotency in embryonic stem cells (ESCs) [3]. Therefore, the regulation of Sox2 is most likely critical for the generation of iPSCs. One enzyme that has been demonstrated to post-translationally regulate Sox2 is the ADP-ribosyltransferase diphtheria toxin-like 1 (Artd1, formerly called Poly(ADP-ribose) polymerase 1/Parp1). Artd1 is a chromatin associated factor that catalyzes the covalent attachment of poly(ADP-ribose) (PAR) to itself and to other nuclear acceptor proteins [4,5]. ADP-ribosylation plays an important role in numerous biological processes, such as maintenance of genomic stability, cell differentiation, cell death, replication, and transcriptional regulation [6,7]. Different roles of Artd1 in the regulation and maintenance of pluripotency have also been previously described: deletion of Artd1 in ESCs globally affects gene expression patterns and

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Artd1 knockout ESCs differentiate into trophoblast derivatives [8,9]. Furthermore, it was previously shown that Artd1-dependent PARylation of Sox2 induces its eviction from the *Fgf4* (fibroblast growth factor 4) enhancer and thereby induces *Fgf4* transcriptional activation [10]. In contrast, recently Lai et al. reported that in ESCs, Artd1 PARylates itself and thereby enhances its interaction with Sox2, which in turn prevents Sox2 from binding to Oct4/Sox2 enhancers [11]. Previous work indicates an important role of Artd1 during reprogramming. Artd1 knockout (Artd1−/−) fibroblasts exhibit impaired reprogramming capacity [11], but the mechanisms underlying this observation were not analyzed. In a recent work, Doege et al. describe a role of Artd1 in conjunction with 10–11 translocation-2 (Tet2) in mediating the histone modifications necessary for the establishment of an activated chromatin state at pluripotency loci [12].

In this study, we aimed at analyzing the role of Artd1 during reprogramming, paying special attention to its role in the first days upon transduction (days 0–4) of the cells with the Yamanaka factors. We found that PARylation of Sox2 by Artd1 between day 0 and day 4 plays an important role in the generation of iPSCs. Inhibition of the enzymatic activity of Artd1 during this time period in wild-type (WT) fibroblasts resulted in a strongly decreased reprogramming efficiency after retroviral-mediated transduction of the Yamanaka factors. The same could be observed when using fibroblasts deficient for Artd1 (Artd1−/−). Our data further show that Artd1-mediated PARylation of Sox2 is involved in the regulation of *Fgf4* expression. The importance of Fgf4 during the first steps of reprogramming is also corroborated by our finding that addition of exogenous Fgf4 can rescue the reprogramming deficiency of the Artd1−/− cells.

In conclusion, our data clearly indicate a new role of Artd1 in regulating Fgf4 activity via Sox2 ADP-ribosylation during reprogramming and suggest a dual function of Artd1 during this process. Artd1 is essential for starting the Fgf4-mediated reprogramming process and later establishes the post-translation modification necessary for the activation of pluripotency genes [12].

Materials and Methods

Reprogramming

Mouse embryonic fibroblasts (MEF) were isolated from 14.5-day-pregnant C57BL/6 mice and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (PAA) and 1% L-glutamin/penicillin/streptomycin (10,000 U/ml penicillin G sodium; 10,000 μg/ml streptomycin sulfate; 29.2 mg/ml L-glutamine; 10 mM sodium citrate in 0.14% NaCl, Gibco, Invitrogen, Basel, Switzerland, www.invitrogen.com). The reprogramming of the MEFs was performed according to Yamanaka's protocol [13] using the pMXs retroviral vectors producing murine *Oct4*, *Sox2*, *Klf4*, and *c-Myc* (Addgene, cat. nos. 13366, 13367, 13370, and 13375). Two days after infection, MEFs were cultured in DMEM containing 15% fetal bovine serum, 1% L-glutamin/penicillin/streptomycin, 1× MEM nonessential amino acids (Gibco, Invitrogen, Basel, Switzerland, www.invitrogen.com), and 50 mM ß-mercaptoethanol (Gibco, Invitrogen, Basel, Switzerland, www.invitrogen.com) supplemented with 1,000 U/ml ESGRO murine Leukemia inhibitory factor (Millipore, Chemikon, Zug, Switzerland, www.millipore.com). FGF4 (Sigma, Buchs, Switzerland, www.sigmaaldrich.com/switzerland-schweiz.html) was added during the reprogramming process at 10 ng/ml unless stated otherwise, ABT-888 (Enzo Life Sciences, New York, www.enzolifesciences.com) at 10 μM and SU5402 (Millipore, Calbiochem, Zug, Switzerland, www.millipore.com) at 2 μM.

Immunofluorescence Staining iPSCs

For immunofluorescence staining, iPSCs derived from WT, Artd1−/− fibroblasts, and Artd1−/− fibroblasts reprogrammed in the presence of Fgf4 (Artd1−/−*) were grown on mitomycin C-treated MEFs and fixed in 4% paraformaldehyde. Then, iPSCs were incubated with primary antibodies against Oct4 (rabbit anti-Oct4, Santa Cruz Biotechnology, Santa Cruz, CA, www.scbt.com) and SSEA-1 (mouse anti-SSEA-1, Millipore). Secondary fluorescence-labeled antibodies were used for detection (goat anti-rabbit Alexa Fluor 594 and goat anti-mouse Alexa Fluor 488, Molecular Probes, Invitrogen, Basel, Switzerland, www.invitrogen.com). Nuclei of the cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Roche, Basel, Switzerland, www.roche.ch).

Real-Time PCR

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Venlo, Netherlands, www.qiagen.com) and 1 μg of total RNA was reverse transcribed with Oligo-dT primers (Invitrogen) and Superscript III (Invitrogen). Real-time PCR was performed in triplicates in a Rotor-Gene Q RG-6000 (QIAGEN) with Rotor-Gene SYBR green (QIAGEN) and analyzed with the Delta Ct-method. GAPDH was used for normalization. Error bars represent the SD of the mean of triplicate reactions. Primers are listed in Supporting Information Table S1.

In Vitro Differentiation

For monoculture neural and smooth muscle differentiation, iPSCs (WT, Artd1−/− and Artd1−/− cells reprogrammed in the presence of Fgf4 [Artd1−/−*]) were plated onto gelatinized 35 mm dishes. The iPSCs were cultivated for 10 days with neural differentiation medium (DMEM/F-12 [Gibco, Invitrogen, Basel, Switzerland, www.invitrogen.com], N2 [1:100, Gibco, Invitrogen, Basel, Switzerland, www.invitrogen.com], B27 [1:50, Gibco, Invitrogen, Basel, Switzerland, www.invitrogen.com], and 1% L-glutamin/penicillin/streptomycin) or smooth muscle differentiation medium (DMEM and 10% fetal bovine serum). At day 10, cells were fixed in 4% paraformaldehyde and stained for βIII-tubulin (Sigma) and smooth muscle actin (Sigma), respectively.

Western Blotting

Cells were collected in radioimmunoprecipitation RIPA buffer (50 mM Tris-HCl pH 8; 400 mM NaCl; 0.5% Nonidet P40; 1% Na-Deoxycholate; 0.1% SDS; Protease Inhibitor Cocktail Tablet, EDTA-free Roche, IN). Proteins were identified by SDS-PAGE (10% acrylamide) and Western blotting using the following antibodies: α-PAR ALX-210–890 (Enzo Life Sciences, New York, www.enzolifesciences.com), α-PARP-1/2 (H250) sc-7150, α-Sox2 15830 (Abcam, Cambridge, United Kingdom, www.abcam.com), α-Pcna (PC10) sc-56, α-tubulin T6199 (Sigma), IRDye 800CW anti-Rabbit, and IRDye 680RD anti-Mouse (LI-COR, Lincoln, Nebraska, www.licor.com). Images were acquired with an Odyssey Imaging System (LI-COR).

In Vitro Sox2 ADP-Ribosylation

HEK293 cells were seeded at a density of 2.7×10^6/150 mm dish and after overnight incubation transfected with pBluescript II and pCAG-HA-Sox2-IP (cat. no. 13459) vectors, respectively (using $CaCl_2$ transfection). After 72 hours, cells were harvested and resuspended in NE buffer (50 mM Tris-HCl [pH 7.5], 0.15 M KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 20% [vol/vol] Glycerol). After sonication at 4°C for 2 × 30-second, the cells were incubated with DNase (Fermentas, Thermo Fisher Scientific Waltham, Massachusetts, http://www.thermoscientificbio.com) for 30 minutes at 4°C. After DNA digestion, the cells were sonicated for 30 seconds and then centrifuged at 6,000 rpm for 10 minutes. The cleared lysate was subjected to immunoprecipitation overnight at 4°C using immobilized antibody against HA (ANTI-HA affinity gel, Sigma). Precipitates were washed three times with

NE buffer and after centrifugation; the HA-Sox2 coupled beads were resuspended in reaction buffer (50 mM Tris-HCl pH8, 4 mM $MgCl_2$, 0.25 mM dithiothreitol, 5 mM NaCl, 200 nM EcoRI linker). Recombinant human ARTD1 (10 pmol) and ^{32}P-NAD+ (4 nmol) were added and the reactions were incubated for 15 minutes at 30°C. Empty pBlueScript II vector was used as a negative control and 0.1 μg of histone H1 (10223549001; Roche) as a positive control. Proteins were resolved by SDS-PAGE (10% acrylamide), exposed to x-ray film (Tx-RP) and analyzed (Typhoon imager, GE Healthcare Life Sciences, Switzerland, http://www.gelifesciences.com).

High Stringency Immunoprecipitation

At day 4, cells were collected in cold phosphate buffered saline and lysed in hypotonic buffer (5 mM Hepes pH 7.5; 85 mM KCl; 0.5% Nonidet P40; protease inhibitor Roche), and nuclei were spun down for 10 minutes at 8,000 rpm. Nuclei were then suspended in High Stringency Buffer (50 mM Tris-HCl pH 7.5; 0.4 M NaCl; 1% Nonidet P40; 0.4% Na-Dedoxycholate) followed by sonication and DNA digestion with DNaseI (Roche). Extracts were cleared by 10 minutes centrifugation at 14,000 rpm. Cleared nuclear extracts were diluted 1:2.7 in 50 mM Tris-HCl pH 7.5 and immunoprecipitations were carried out for 2 hours using 10H antibody or IgG as a negative control. Beads were then washed three times in the same buffer and lastly boiled in SDS loading buffer.

Immunofluorescence Staining During Reprogramming

WT and Artd1−/− fibroblasts were seeded on glass coverslips and reprogramming was induced as previously described. At the indicated time, cells were fixed in acetic acid/methanol (1:3) for 5 minutes on ice, blocked for 30 minutes in PBSMT (phosphate buffered saline containing 5% milk and 0.05% Tween-20), incubated with α-Sox2 (15830, Abcam, 1:200) and α-PAR (10H, Enzo Life Sciences, New York, www.enzolifesciences.com) (1:250) dissolved in PBSMT for 1 hour at room temperature, washed with phosphate buffered saline, incubated with secondary Alexa Fluor 488 α-rabbit (Invitrogen) and Cy3-IgG fraction monoclonal mouse anti-FITC antibodies, and embedded on microscopy slides with DAPI containing mounting medium VECTASHIELD. Images were acquired with a Leica SP5 microscope at the Centre for Microscopy and Image Analysis of the University of Zürich.

Sox2 Coimmunoprecipitation

WT and Artd1−/− fibroblasts were reprogrammed as previously described. At the indicated time, cells were harvested and lysed in hypotonic buffer (5 mM Hepes, 85 mM KCl, 0.5% Nonidet P40, Protease Inhibitor [Roche]). Nuclei were pelleted at 8,000 rpm for 10 minutes at 4°C and resuspended in immunoprecipitation buffer (50 mM Tris-HCl pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, protease inhibitor [Roche], 0.5 mM dithiothreitol), sonicated, and DNA was digested with DNaseI (Roche). Immunoprecipitations were carried out using 200 μg of nuclear proteins and 2 μg of α-Sox2 (15830, Abcam) for 2 hours at 4°C, followed by three washes in washing buffer (20 mM Tris-HCl pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 10% glycerol, 0.1% Tween-20, protease inhibitor [Roche]), and eventually resuspended in 1× SDS-loading buffer.

Chromatin Immunoprecipitation

WT, Artd1−/−, and ABT-888 inhibited cells were crosslinked with 1% formaldehyde (Calbiochem). Chromatin was fragmented with the Bioruptor (Diagenode, Liège, Belgium, http://www.diagenode.com), incubated with specific antibodies, and collected with Protein A Agarose/salmon sperm DNA (Millipore). DNA was extracted and measured by real-time PCR using SYBR Green and Rotor-Gene 3000 (Corbett Life Science/QIAGEN). For Primer sequences see Supporting Information Table S1.

Results

Artd1 Is Necessary for Successful Initiation of Reprogramming

MEFs were isolated from day 14 embryos obtained from homozygous breeding of WT and Artd1−/− mice, respectively. WT and Artd1−/− fibroblast were transduced with the Yamanaka factors as previously described [13] and the number of iPSC-colonies was assessed after 14 days of cultivation. The number of iPSC colonies obtained upon reprogramming of Artd1−/− fibroblasts was reduced by around 65% compared to WT fibroblasts (Fig. 1A). These observations are in agreement with recently published data [11] and indicate that Artd1 is required for the reprogramming of somatic cells to iPSCs. An even stronger reduction in the number of iPSC colonies (80%) was observed when the PARP-Inhibitor ABT-888, which mainly inhibits Artd1 and Artd2 [14], was applied. The small difference in the number of colonies between cells lacking Artd1 and PARP-inhibitor treated cells might indicate that the contribution of other ARTD family members than Artd1 during reprogramming is minimal. In addition, the ABT-888 inhibitor did not change the transcriptional levels of Artd1 and Artd2, indicating that the effects observed are exclusively due to the inhibition of the enzymatic activity (Supporting Information Fig. S1A, S1B). In order to define if ADP-ribosylation is necessary during the whole reprogramming process or only during a specific time window, we added the ABT-888 inhibitor starting from days 0, 2, 4, 6, and 8 after the transduction of WT fibroblasts with the Yamanaka factors. iPSC colony formation was strongly reduced when the inhibitor was added during the first 4 days of reprogramming, but unaffected if cells from later time points after viral infection were treated (Fig. 1B), indicating that Artd1 enzymatic activity is essential during the early phase of the reprogramming process. In order to determine the expression changes of Artd1 and Artd2 during the first 12 days of reprogramming, we performed quantitative real-time PCR analysis (Fig. 1C, 1D). The expression of both Artd1 and Artd2 constantly increased from day 2 to day 8 and dropped to levels similar to the untreated control cells by day 12. This increase in Artd1 expression was also observed at the protein level (Fig. 1E) and was in accordance with increased PARylation in fibroblasts at day 2, followed by a decrease at day 4 and a constant level in the following days (Fig. 1F). In summary, our data indicate an upregulation of Artd1 and Artd2 expression and of PARylation during the first days of reprogramming. Blocking of Artd1, either by genetic ablation or by applying a PARP-inhibitor, drastically decreases the efficiency of reprogramming.

Artd1 Is Responsible for Poly(ADP-Ribosylation) of Sox2 During Reprogramming

In ESCs, Artd1 was previously described to PARylate Sox2, thereby decreasing the association of Sox2 with the *Fgf4* enhancer and inducing Fgf4 expression [10]. In order to test the capacity of Artd1 to PARylate Sox2, we transfected a HA-Sox2 expression vector in HEK293 cells and the recombinant protein was purified by immunoprecipitation. Upon in vitro incubation of the purified protein with recombinant human ARTD1 and radiolabeled NAD^+, a signal at the predicted size of Sox2 was clearly detected, indicating that Sox2 is substrate of ARTD1 (Fig. 2A). To further prove that Sox2 is PARylated during reprogramming, WT, WT+ABT-888, and Artd1−/− day 4 cells were collected in high stringency ionic buffer in order to reduce cellular protein complexes. Cleared nuclear lysates were subsequently used to immunoprecipitate PARylated proteins with an anti-PAR antibody. PARylated

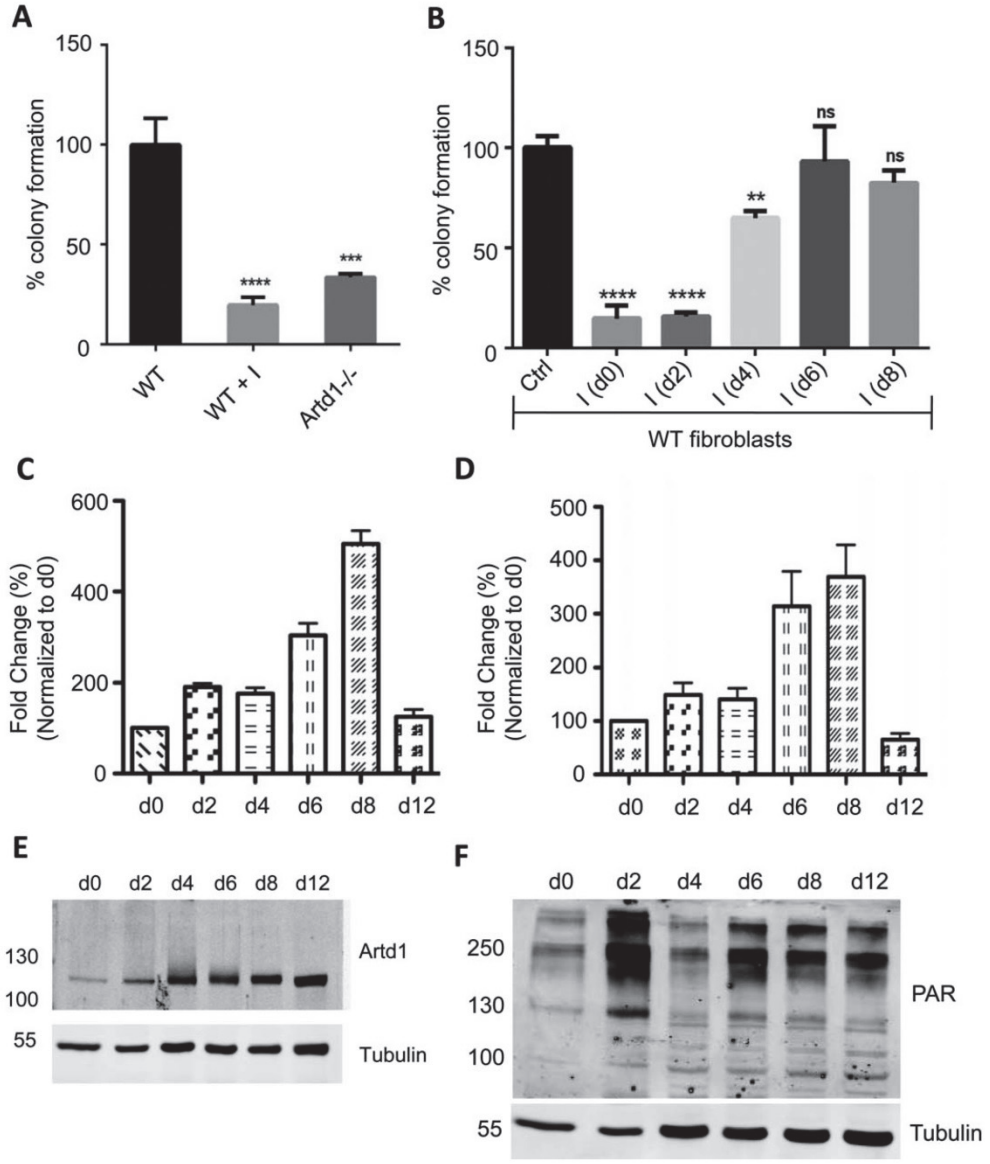

Figure 1. Artd1 enzymatic activity is necessary for the initial steps of reprogramming. **(A):** Reprogramming efficiency in WT fibroblasts, Artd1−/− fibroblasts, and WT fibroblasts treated with ABT-888. **(B):** Artd1 enzymatic activity is essential during the early phase of the reprogramming process. ABT-888 was added at the indicated time points and the reprogramming efficiency was assessed. **(C):** Expression of Artd1 in WT cells during the reprogramming process. **(D):** Expression of Artd2 in WT cells during the reprogramming process. **(E):** Expression levels of Artd1 during reprogramming. Western blot for Artd1 on WT cells collected at the indicated time points during reprogramming. The smear of the Artd1 signal reflects Artd1 activity. Tubulin has been used as a loading control. Molecular size references in kilo Daltons are indicated. **(F):** PAR formation during reprogramming. Western blot for PAR on WT cells collected at the indicated time during reprogramming. Tubulin has been used as a loading control. Molecular size references in kilo Daltons are indicated. Dunnett's multiple comparison test. ns = not significant, **, $p < .01$; ***, $p < .001$; ****, $p < .0001$. Abbreviations: PAR, poly(ADP-ribose); WT, wild type.

proteins were resolved in SDS-PAGE, followed by Western blotting against Sox2 and Artd1. Sox2 as well as Artd1 were pulled down in WT extract but not in WT+ABT-888 or Artd1−/− extracts, indicating that Sox2 is a targeted by ADP-ribosylation in vivo. (Fig. 2B) In order to assess the expression pattern of Sox2 and the PARylation levels during the first phase of reprogramming, we performed Western blotting of Sox2 (Supporting Information Fig. S2) and monitored the localization of Sox2 and PAR by immunofluorescence in WT and Artd1−/− fibroblast at days 0, 2, 4, and 6 after viral infection with the Yamanaka factors (Fig. 2C and Supporting Information Fig. S3). In the nuclei of WT fibroblasts PARylation was detectable starting from day 2 post-transduction and was still present at day 6. Interestingly, Sox2 staining mainly colocalized with the PAR signal, suggesting that ADP-ribosylation of Sox2 occurs at the beginning of reprogramming. As expected, a PAR signal was not detectable in the Artd1−/− fibroblasts at any time point, thus supporting the idea that Artd1 is the major ADP-ribosyltransferase involved in the process.

In order to clarify if in vivo, during the first phases of reprogramming, Artd1 and Sox2 directly interact, WT and Artd1−/− fibroblasts were transduced with the Yamanaka

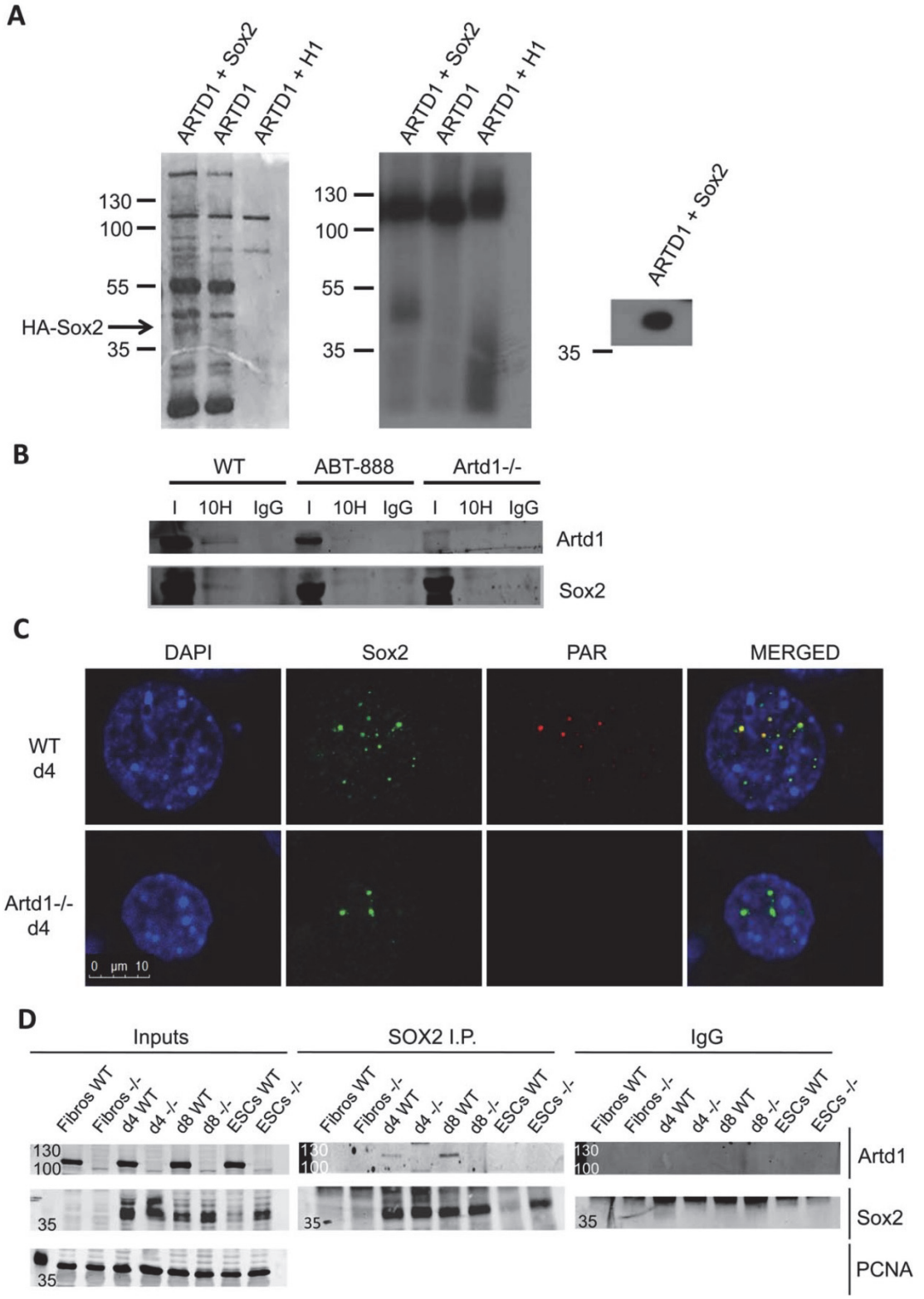

Figure 2. Artd1 binds and post-translationally modifies Sox2 during reprogramming. **(A):** Trans ADP-ribosylation of HA-Sox2 by recARTD1. Recombinant human ARTD1 (10 pmol) was incubated with HA-Sox2 (lane 1), HA-empty (lane 2), or H1 as positive control (lane 3). Coomassie blue stained gel (left), autoradiography (middle), and Western blot for Sox2 bound to the beads (right) are shown. **(B):** Sox2 ADP-ribosylation during reprogramming. High stringent immunoprecipitation was carried on with either PAR antibody 10H or IgG control on nuclear extracts of WT, ABT-888 treated WT, and Artd1−/− cells collected at day 4. Western blots of Sox2 and Artd1 in immunoprecipitation samples and inputs are shown. **(C):** Immunofluorescence of Sox2 and PAR during reprogramming at day 4. WT and Artd1−/− cells were stained with DAPI, for Sox2 and for PAR. Signals in the relative channels are shown from left to right. Merge of the three channels is also reported. Scale bar = 10 µm. **(D):** Sox2/Artd1 interaction during reprogramming. Immunoprecipitation was carried on with either Sox2 antibody or IgG control on nuclear extracts of WT and Artd1−/− cells collected at the indicated time. Western blots of Sox2 and Artd1 in immunoprecipitation samples and inputs are shown. proliferating cell nuclear antigen (PCNA) was used as indicator of equal starting protein content for the immunoprecipitations. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; ESCs, embryonic stem cells; Fibros, fibroblasts; WT, wild type.

factors and protein extracts were isolated at day 4 and day 8 upon transduction. Artd1/Sox2 coimmunoprecipitation was detectable at day 4 and day 8 in WT fibroblasts, clearly indicating that these two proteins interact in reprogramming fibroblasts. As expected, in Artd1−/− fibroblasts, no interaction was detectable (Fig. 2D). Taken together, our data show that within the first 8 days of reprogramming, Artd1 interacts with Sox2 and mediates its PARylation.

Artd1-Mediated ADP-Ribosylation of Sox2 Is Responsible for the Activation of Fgf4 Transcription

Although Sox2 has been reported to bind to the *Fgf4* enhancer element, its effect on *Fgf4* transcription remains controversial. Originally, it was described that Sox2 binding enhances *Fgf4* transcription [15]. In contrast, a more recent study reported that in ESCs and in differentiating cells, Sox2 represses *Fgf4* transcription and that ADP-ribosylation of Sox2 relives Fgf4 repression [10]. In addition, the work of Lai et al. showed that in ESCs, Sox2 positively regulates *Fgf4* transcription and that auto-modified Artd1 interacts with Sox2, consequently inducing its release from the *Fgf4* enhancer and repressing *Fgf4* transcription [11]. To investigate the role of Sox2, Artd1, and ADP-ribosylation in the fine-tuning of *Fgf4* transcription, we first analyzed the expression of *Fgf4* upon initiation of reprogramming. In WT fibroblasts, expression of *Fgf4* was detectable starting from day 2 and increased steadily until day 6. In contrast, in Artd1−/− as well as in WT fibroblasts treated with the ABT-888 inhibitor, expression was strongly reduced and delayed. This clearly indicates that the presence of Artd1 is necessary for the correct activation of *Fgf4* transcription (Fig. 3A). In order to assess the binding capacity of Sox2 to the *Fgf4* enhancer, we performed ChIPs using antibodies against Sox2 during reprogramming in WT, Artd1−/−, and in WT fibroblasts treated with ABT-888. As depicted in Figure 3B, during the reprogramming process, Sox2 is recruited to the *Fgf4* enhancer and to other target sites such as the *Nanog* promoter. In ABT-888 inhibited fibroblasts, Sox2 recruitment is delayed, which is in agreement with the lower transcription of *Fgf4* (Fig. 3A), indicating that PARylation positively influences the DNA binding capacity of Sox2. Strikingly, the delay of Sox2 recruitment in the presence of ABT-888 is phenocopied in Artd1−/− cells (Fig. 3B), confirming that Artd1 is mainly responsible for Sox2 ADP-ribosylation in reprogramming cells. Taken together, our data demonstrate that ADP-ribosylation of Sox2 strengthens the binding of Sox2 to its target sites and thereby stimulates the transcription of the corresponding target genes.

Fgf4 Expression Is Crucial for the Initiation of Reprogramming

The reduced transcription of *Fgf4* in Artd1−/− fibroblasts upon the initiation of reprogramming leads to the question if the impaired reprogramming efficiency of Artd1−/− cells is the direct consequence of the reduced Fgf4 levels or if other factors are involved. We therefore repeated the reprogramming experiments in WT and Artd1−/− fibroblast by adding 10 ng/ml and 25 ng/ml of exogenous Fgf4 to the cells. Addition of 10 ng/ml of Fgf4 was sufficient to restore the reprogramming efficiency to comparable levels as in WT cells (Fig. 4A), indicating that the phenotype observed in Artd1−/− cells is due to an insufficient expression of Fgf4 during the early stages of reprogramming. Interestingly, higher amounts of Fgf4 (25 ng/ml) or addition of exogenous Fgf4 to WT fibroblast rather impaired the reprogramming efficiency, suggesting that a tight control of autocrine Fgf4 production is essential for the initiation of reprogramming. The Artd1−/− iPSC colonies obtained upon addition of Fgf4 could be expanded for more than 10 passages and expressed the classic pluripotency genes. Furthermore, they were able to differentiate in vitro toward smooth muscles and neurons and showed no differences from WT and Artd1−/− iPSCs (Supporting Information Fig. S4).

The fact that Fgf4 transcription depends on ADP-ribosylation of Sox2 and the capacity of exogenous Fgf4 to restore the reprogramming efficiency in Artd1−/− fibroblasts suggests that the enzymatic activity of Artd1 is essential for the initiation of reprogramming. To test this, we reprogrammed WT fibroblasts and cultivated the cells for the first 2, 4, or 6 days with ABT-888 or with ABT-888 and 10 ng/ml Fgf4. The addition of Fgf4 for the first 2 or 4 days abolished the inhibitor effect of ABT-888 and significantly increased the reprogramming efficiency (Fig. 4B), indicating that the direct addition of Fgf4 compensates for the absence of ADP-ribosylation activity. This strengthens the observation that Artd1-mediated ADP-ribosylation of Sox2 is essential to modulate Fgf4 transcription during the initial phases of reprogramming. To further prove the importance of Fgf4, we performed Fgf4 knockdown experiments. The combination of viral transduction of the reprogramming factors with knockdown induced massive cell death in the fibroblast. This was not due to Fgf4 knockdown itself because scrambled controls had the same effect. We therefore decided to inhibit Fgf receptor tyrosine kinases with small chemical inhibitor SU5402 [16] in WT cells. The inhibition of Fgf receptor tyrosine kinases reduced the reprogramming efficiency of WT fibroblasts by around 50% (Fig. 4C). In summary, our results clearly identify Artd1-mediated ADP-ribosylation of Sox2 as an essential component for the correct activation of Fgf4 expression, which in turn plays a crucial role for the initiation of the reprogramming process.

Discussion

Cell differentiation is normally an irreversible process and differentiated cells are not able to switch from one lineage to another. Cellular reprogramming to pluripotency therefore requires that pluripotency genes, which are inactive in differentiated cells, are reactivated. Exogenously introduced reprogramming factors must therefore bind and reactivate their target genes in tight collaboration with other endogenous factors, particularly epigenetic regulators.

In this work, we aimed at understanding the function of the well-known epigenetic regulator Artd1 during the first phase of reprogramming. Artd1 was previously shown to be necessary for proper differentiation of ESCs [8]. Even though Artd1 deficiency does not affect the growth of ESCs, its absence compromises cell survival and growth when ESCs are induced to differentiate [8,10]. The molecular mechanisms underlying this observation are still largely unclear, but new studies indicate that Artd1 acts as a cofactor of Oct4 and Sox2 in ESCs by binding to the *Fgf4* enhancer and thereby regulates Fgf4 expression [10,11]. The major function of Fgf4 in pluripotent cells is to regulate the selection between the alternative fates of self-replication and lineage commitment during continuous proliferation. Autocrine production of Fgf4 is the major stimulus activating the Erk1/2 signaling cascade in naïve mouse ESCs [17,18]. Inhibiting ERK and FGF activity with small chemical compounds prevents ESCs from differentiating without affecting the propagation of the

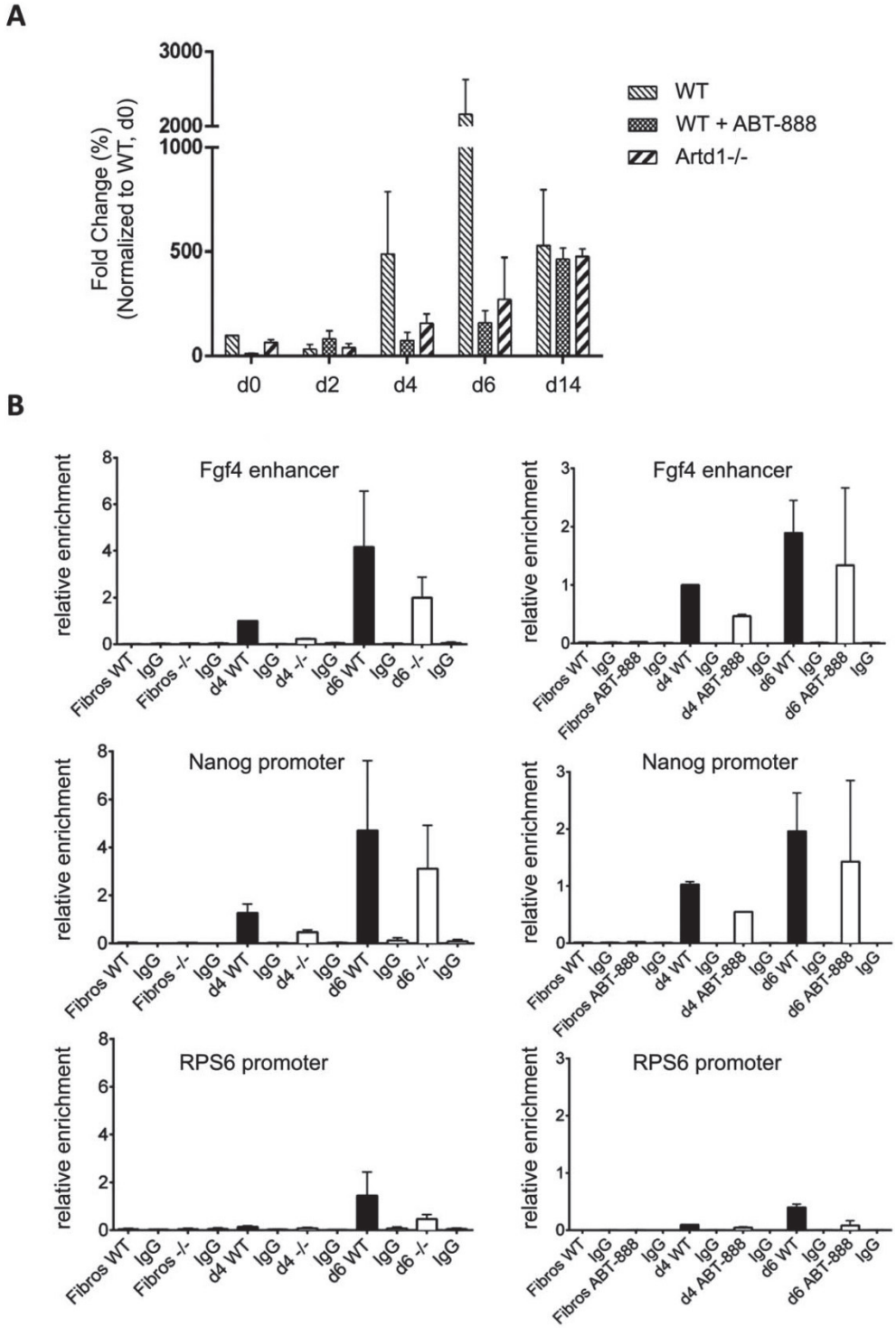

Figure 3. Artd1 activity is necessary for the binding of Sox2 to the Fgf4 enhancer and for driving Fgf4 expression. **(A):** Expression of Fgf4 during the reprogramming process in WT fibroblasts ± ABT-888 and Artd1−/− fibroblasts. **(B):** Recruitment of Sox2 to target genes: chromatin immunoprecipitation was carried on with either Sox2 antibody or IgG control in WT, Artd1−/−, and ABT-888 inhibited cells at the indicated time points. Recruitment of Sox2 to the Fgf4 enhancer, the Nanog promoter, and the unrelated Rps6 promoter is depicted. Values are expressed as enrichment over input signals. Abbreviation: WT, wild type.

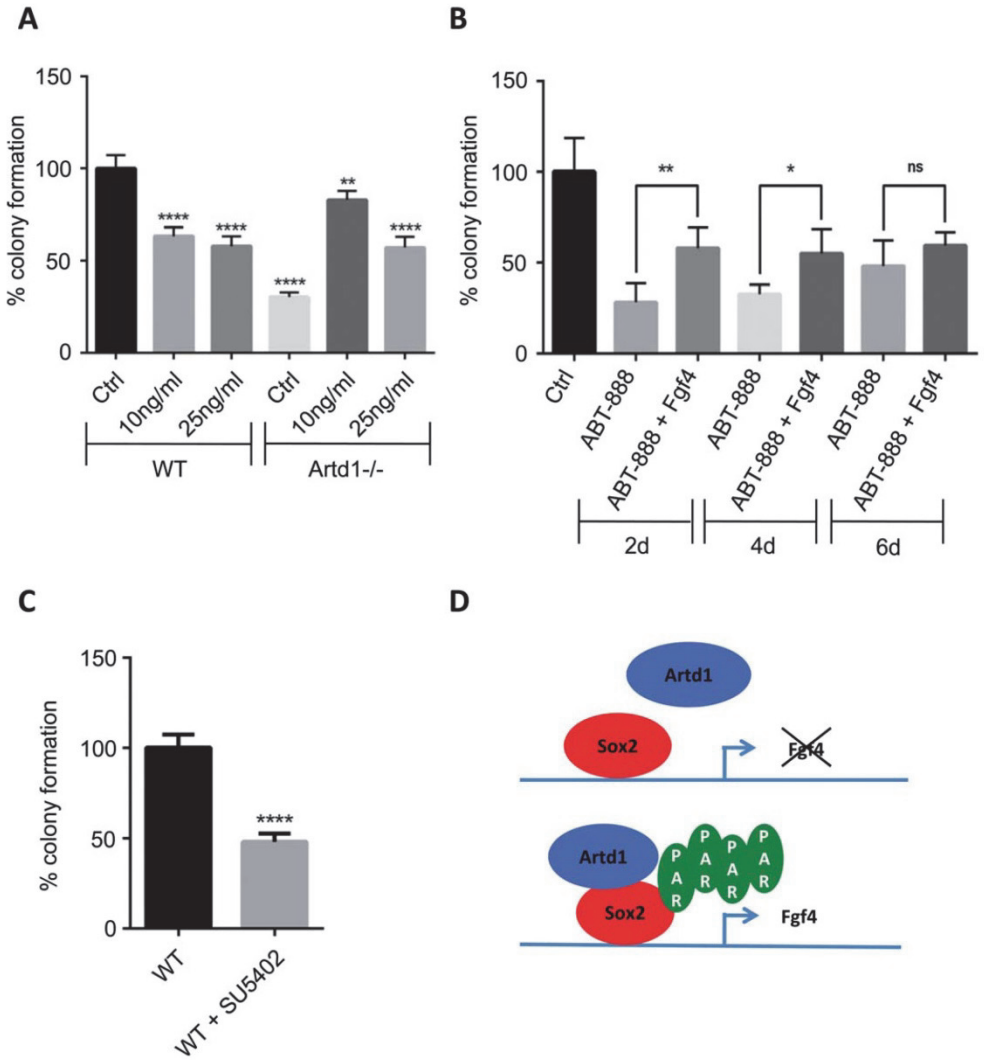

Figure 4. Exogenous Fgf4 supplementation is sufficient to restore the reprogramming capacity in Artd1−/− fibroblasts and WT cells treated with ABT-888 inhibitor. **(A):** Reprogramming efficiency of Artd1−/− cells cultivated with exogenously added Fgf4. **(B):** Reprogramming efficiency of WT+ABT-888 cells in the presence or absence of Fgf4. WT fibroblasts were treated for the first 2, 4, or 6 days with ABT-888 alone or ABT-888 and 10 ng/ml Fgf4. **(C):** Reprogramming efficiency in WT cells and WT cells treated with SU5402, an inhibitor of Fgf receptor tyrosine kinase activity. **(D):** Schematic representation of Artd1-mediated PARylation of Sox2 and binding to the Fgf4 enhancer, which activates Fgf4 expression. Dunnett's multiple comparison test (A) and Student's *t* test (B, C). ns = not significant, *, $p > .05$; **, $p < .01$; ****, $p < .0001$. Abbreviation: WT, wild type.

undifferentiated ESCs [18,19]. Similarly, ESCs lacking Fgf4 are resistant to neural and mesodermal induction, but are able to commit when FGF is provided exogenously [18].

During differentiation of ESCs, Artd1 was shown to directly interact with and to PARylate Sox2, leading to the dissociation and degradation of Sox2 from the *Fgf4* enhancer. This releases Sox2 inhibition and induces *Fgf4* gene transcription [10]. In the absence of activated Artd1, Sox2 cannot be ADP-ribosylated, augmenting its interaction with the *Fgf4* enhancer and leading to a stabilization of Sox2 protein and a reduction in Fgf4 levels [10]. An alternative model suggests that Artd1 auto-PARylation enhances Sox2-Artd1 interactions and inhibits binding of Sox2 to the Oct4/Sox2 site at the *Fgf4* enhancer. This process seems to be regulated by FGF/ERK signaling [11].

We first tested the reprogramming capacity of Artd1 knockout cells and found that the absence of Artd1 strongly reduces the number of iPSC colonies. We could also identify that the critical time period during which Artd1 activity is necessary are the first 2–4 days after transduction with the reprogramming factors. This is in agreement with the increased expression of Artd1 starting at day 2 after reprogramming and the concomitant increase of PARylation. The reduction of the reprogramming efficiency is mainly due to the lack of Artd1 activity and not of other members of the ARTD family, because the treatment of WT fibroblasts with ABT888, an inhibitor of poly(ADP-ribosyltransferases), mimics the genetic ablation of Artd1. We further observed a strong delay in *Fgf4* expression upon the initiation of reprogramming in Artd1−/− fibroblasts or when WT fibroblasts are treated with ABT-888. Fgf4 expression occurs much earlier than the activation of transcription of other typical pluripotency markers such as Nanog, SSEA-1, or OCT-4. This observation is interesting because Fgf4 is typically expressed in pluripotent cells [18,20,21] and not in fibroblasts.

Based on previous studies [10,11] indicating a role of Artd1 in modulating Sox2 activity in the context of Fgf4 regulation, we decided to analyze the capacity of Artd1 to modify Sox2 in vitro and in vivo. Our data demonstrate that human ARTD1 is able to PARylate Sox2 in vitro and strongly suggest that murine Artd1 mediates ADP-ribosylation of Sox2 in vivo in fibroblasts starting from day 2 during reprogramming. The role of Sox2 in the regulation of *Fgf4* transcription is also highlighted by the observation that ADP-ribosylation of Sox2 increases its binding to the *Fgf4* enhancer and leads to increased transcription. In summary, our data confirm that during the early phase of the reprogramming process, Artd1-mediated ADP-ribosylation of Sox2 is necessary for the binding of Sox2 to the *Fgf4* enhancer and for inducing Fgf4 expression, which in turn is responsible for initiating the further events leading to the formation of iPSCs.

The importance of Fgf4 during the first phase of the reprogramming process is strengthened by the fact that Artd1−/− fibroblasts, which show a strongly reduced activation of *Fgf4* upon reprogramming initiation, show a massive reduction in the number of iPSC colonies. The simple addition of Fgf4 during this time is sufficient to restore the reprogramming efficiency to comparable levels as in WT cells, indicating that Fgf4 is functionally the only factor regulated by Artd1 during the early phase of reprogramming (Fig. 4A). A similar effect can be observed when WT fibroblasts are cultivated in the presence of the ABT-888 inhibitor. Also in this case the simple addition of Fgf4 to the medium is sufficient to restore the reprogramming efficiency (Fig. 4B). The importance of Fgf4 during the early phase of reprogramming is also corroborated by the fact that WT cells treated with an inhibitor of Fgf receptor tyrosine kinases, which are normally activated upon the binding of Fgf, reduces the reprogramming efficiency by 50% (Fig. 4C).

Of interest, Artd1 in conjunction with 10–11 translocation-2 (Tet2) was recently shown to play an important role in the early stages of somatic cell reprogramming by mediating the histone modifications necessary for the establishment of an activated chromatin state at pluripotency loci (e.g., Nanog and Essrb) [12]. Furthermore, Artd1 induction promotes accessibility to the Oct4 reprogramming factor. Interestingly, pluripotency factors are detectable starting around days 10–12 of the reprogramming process [22–25], which would suggest that Artd1 might have two different functions. In the first phase (first week) of the reprogramming process, Artd1 is required for initiating the transcription of Fgf4. In a second step, Artd1 might be involved in promoting the accessibility of the reprogramming factors to the pluripotency gene promoters as shown by Doege et al. [12]. Since we did not observe an effect of PARP inhibitors on the reprogramming efficiency at this time point, it is fair to assume that this process is independent of ADP-ribosylation. Furthermore, our data demonstrate that even in the absence of Artd1, exogenously supplied Fgf4 permits reprogramming efficiencies as for WT fibroblasts, indicating that in the ARDT1−/− cells additional epigenetic modifiers must be interacting with Tet2 to mediate the histone modifications necessary for the activation of pluripotency genes.

Conclusions

Our data indicate that PARylation of Sox2 by Artd1 plays an important role in the generation of iPSCs. Artd1-mediated PARylation of Sox2 favors its binding to the Fgf4 enhancer, thereby activating *Fgf4* expression (Fig. 4D). Exogenous addition of Fgf4 during the first 4 days upon initiation of reprogramming was sufficient to restore the reprogramming capacity of Artd1 knockout fibroblast to WT levels, indicating that Fgf4 is an essential component for the correct initiation of the reprogramming process.

Acknowledgments

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Supplementary Data

Supplemental Figure 1:

Artd1 and Artd2 are expressed during the first days of reprogramming. **A.** Expression of Artd1 during the reprogramming process in WT and Artd1-/- fibroblasts and WT fibroblasts treated with ABT-888. **B.** Expression of Artd2 during the reprogramming process in WT and Artd1-/- fibroblasts and WT fibroblasts treated with ABT-888.

Supplemental Figure 2:

Sox2 expression during reprogramming. **A.** Western Blot analysis for Sox2 in WT and Artd1-/- fibroblasts during the reprogramming process. **B.** Western Blot analysis for Sox2 in WT fibroblasts treated with or without ABT-888 during the reprogramming process.

Supplemental Figure 3:

Colocalization of PAR and Sox2 during the first 6 days of reprogramming. Immunohistochemical localization of Sox2 and PAR in WT and Artd1-/- fibroblasts at d2 and d6 during reprogramming. In addition, WT and Artd1-/- cells were stained with DAPI. Signals in the relative channels are shown from left to right. An overlay of the 3 channels is also reported. Scale bar = 10 μm.

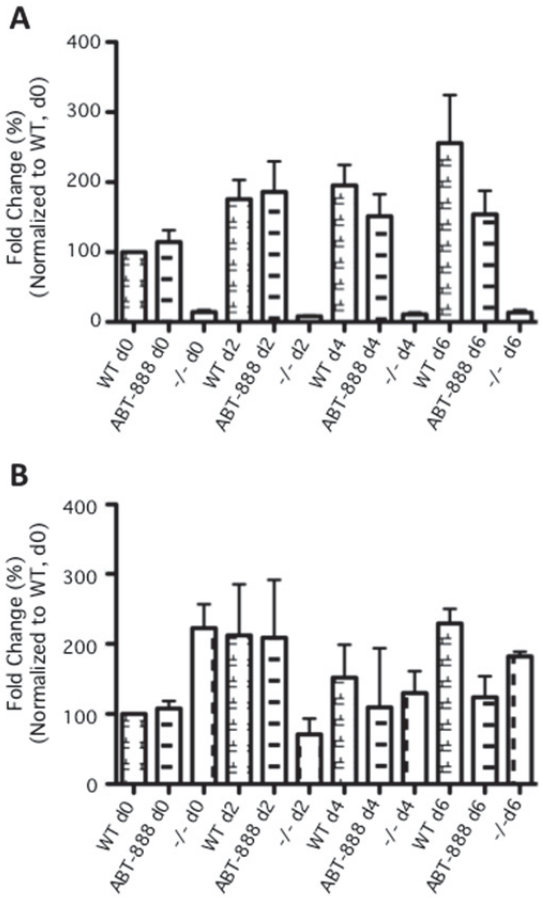
Supplemental Figure 4:

Characterization of WT and Artd1-/- iPSCs and Artd1-/- iPSCs reprogrammed in the presence of 10ng/ml Fgf4 (Artd1-/-Fgf4). **A.** Immunostaining for Oct4 and SSEA-1 in WT, Artd1-/- and Artd1-/-Fgf4 (Artd1-/- cells reprogrammed in the presence of Fgf4) iPSCs. The scale bars represent 100μm. **B.** Immunostaining for SMA and βIII-tubulin for *in vitro* differentiated WT, Artd1-/- and Artd1-/-* iPSCs. The scale bars represent 100μm.

Supplemental Table 1:

Sequences of the primers used.

Supplemental Figure 1



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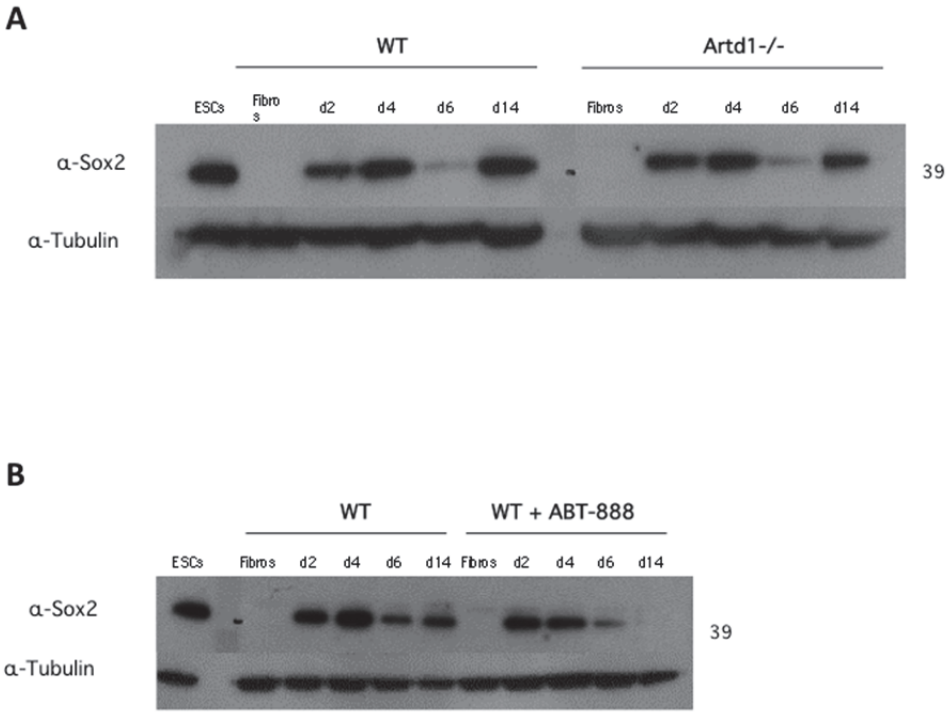

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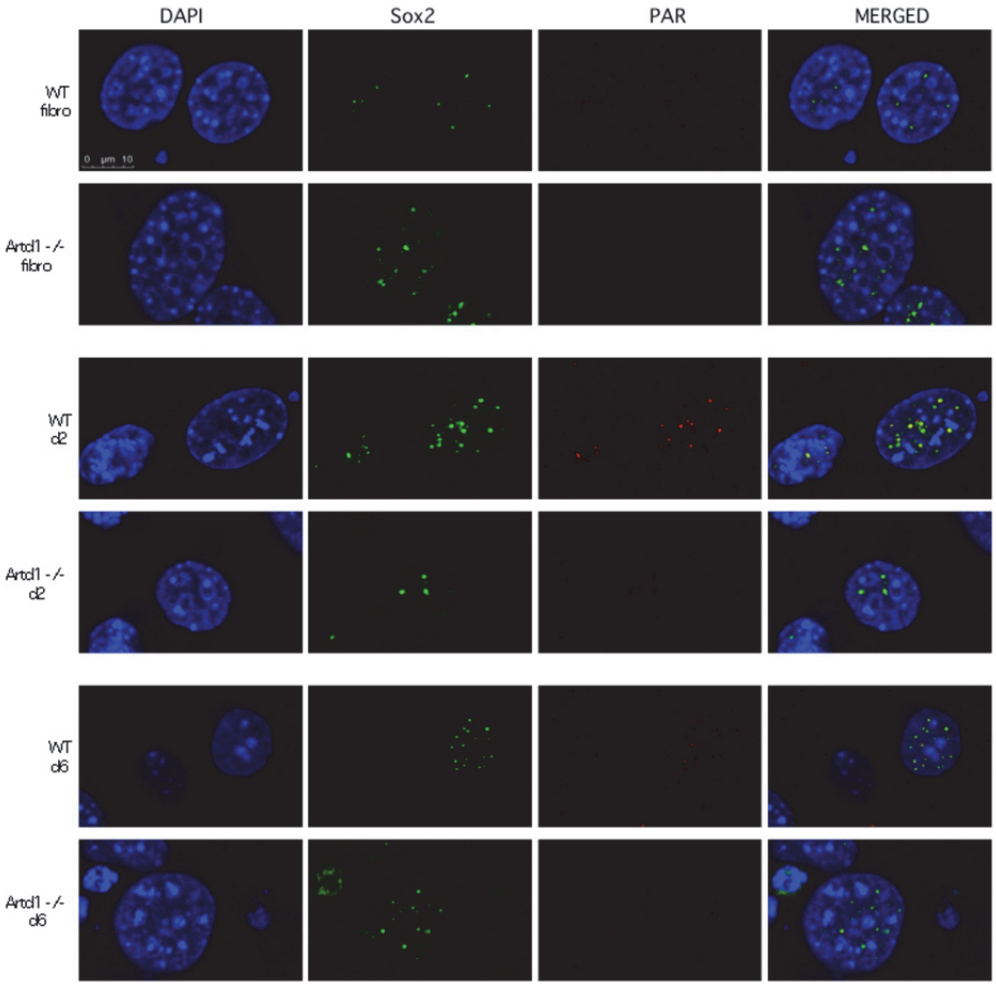
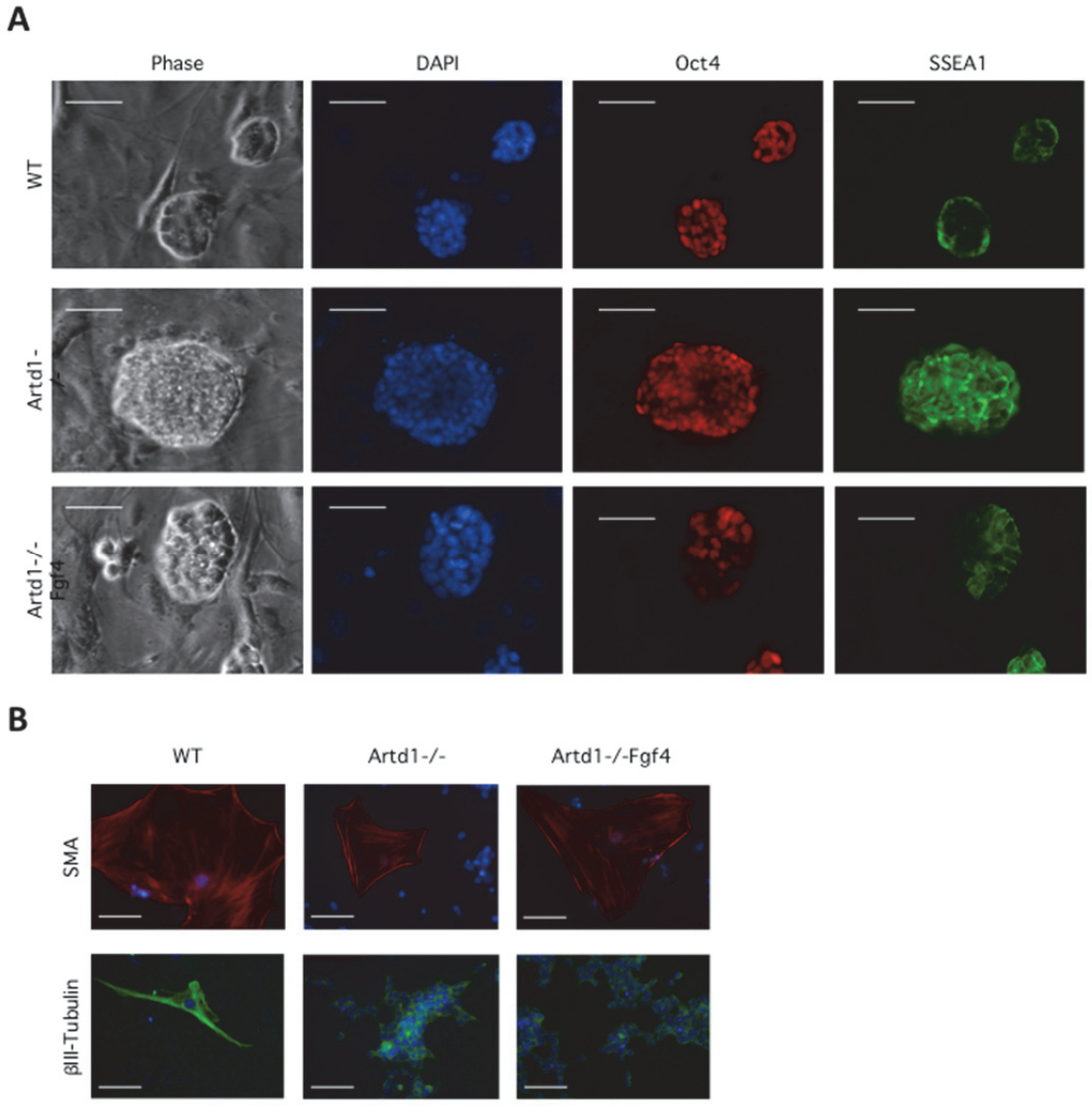


Supplemental Figure 4



Supplemental Table 1

Artd1	Artd1_FWD	5’-GAAGGAAAGAGAAAAGGTGACG-3’
	Artd1_BWD	5’-GCAACTCTGTCCAAGATCGCTG-3’
ARTD2	ARTD2_FWD	5’-GCTGCCAGCACGCAGGATGA-3’
	ARTD2_BWD	5’-AGCGGGGCTCTCTTGGTGTCA-3’
Fgf4	Fgf4_FWD	5’-GCAGCGAGGCGTGGTGAGCATCTT-3’
	Fgf4_BWD	5’-CCCCTTCTTGTTCCGCCCGTTCTT-3’
Fgf4 enhancer	Fgf4_enh_FWD	5’-GCAAGACTGGAAAATCTCATTGGC-3’
	Fgf4_enh_BWD	5’-CATCTTGGGCTGTGGTACAGAATAG-3’
GAPDH	GAPDH_FWD	5’-GCAGCGAGGCGTGGTGAGCATCTT-3’
	GAPDH_BWD	5’- CCCCTTCTTGTTCCGCCCGTTCTT-3’
Nanog promoter	NANOG_Pr_FWD	5’- CCTAAGCTTTCCCTCCCTCC-3’
	NANOG_Pr_BWD	5’- CCCACCAGCCCTGTGAATTC-3’
Rps6 promoter	Rps6_Pr_FWD	5’- GCGTCACGAAAAAGAGGCCCGA-3’
	Rps6_Pr_BWD	5’- AGTCACTTCCGCCGGCTGTTC-3’

Annex 2

Fgf4 controls mesenchymal-to-epithelial transition during the early phases of reprogramming

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Fgf4 controls mesenchymal-to-epithelial transition during the early phases of reprogramming

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Abstract

The ground-breaking discovery that somatic cells can be reprogrammed to induced pluripotent stem cells holds great promise for the study of diseases and therapeutic applications. In the past years a tremendous effort was done to refine the reprogramming technique, but the knowledge about the precise molecular mechanisms underlying the reprogramming process is still limited. An enhanced understanding of the reprogramming process may not only guide the design of improved reprogramming methods, which would lead to increased efficiency, but could also allow the generation of high-quality iPSCs. We could recently show that *Fgf4* expression is regulated by Parp1 and Sox2 during the reprogramming process and plays a crucial role in this process. In this study we aimed at dissecting the underlying mechanism by which Fgf4 modulates the reprogramming process. Fgf4 is typically expressed in pluripotent stem cells such as embryonic stem cells and induced pluripotent stem cells, but absent in fibroblasts. Our data indicate that *Fgf4* expression is starting early upon initiation of reprogramming and constantly increases during the process. Remarkably, the addition of exogenous FGF4 during reprogramming induces an increase in the number of iPSC colonies and inhibition of FGF-signalling negatively affects the reprogramming efficiency. Furthermore, our work identifies Fgf4 for the first time as an important soluble factor for promoting the central phase (d4-d8) of reprogramming. We could show that Fgf4 regulates cell proliferation and favours mesenchymal-to-epithelial transition during this phase via parallel upregulation of epithelial gene expression and downregulation of mesenchymal genes.

Introduction

The possibility of reprogramming somatic cells to a pluripotent state represents one of the most significant discoveries of the recent years and has opened new important perspectives in the field of regenerative medicine [1]. As a consequence it is not astonishing that a number of studies have been performed to optimize this technology (reviewed in [2]). Nevertheless, the molecular mechanisms underlying the reprogramming process are still largely unknown. We recently found that poly(ADP-ribosylation) of the Yamanaka factor Sox2 by poly(ADP-ribose) polymerase 1 (Parp1) plays an important role during the first days upon transduction with the reprogramming factors. We demonstrated that the effect of Sox2 and Parp1 occurs by regulating *Fgf4* expression in the cells which are undergoing reprogramming [3]. We showed that *Fgf4* expression is strongly delayed in Parp1 knockout fibroblasts and in wild type (WT) fibroblasts treated with the PARP-inhibitor ABT-888. In both cases, the delayed expression leads to a massive reduction of the reprogramming efficiency. This early expression of *Fgf4* during reprogramming is intriguing because Fgf4 is typically expressed in pluripotent cells [1, 4-6] and not in fibroblasts. Remarkably, Fgf4 expression during reprogramming precedes the activation of the core pluripotency markers Nanog, SSEA-1, and Oct4 [7].

Fgf4 regulates the decision between the alternative fates of self-replication and lineage commitment in continuously proliferating pluripotent cells. In naïve embryonic stem cells (ESCs) a major role of autocrine Fgf4 is to stimulate differentiation through the activation of the Erk1/2 signalling cascade [4, 8]. This role is underlined by the observation that inhibiting ERK and FGF activity with small chemical compounds prevents ESCs from differentiating without affecting their propagation capacity [4, 9]. Furthermore, when ESCs are lacking Fgf4, they are unable to differentiate toward neural and mesodermal derivatives, a condition which can be reverted by exogenous provision of FGF4 [4]. The role of Fgf4 during reprogramming is largely unknown, but its early activation during the reprogramming process suggests an additional function independent from the roles exerted in pluripotent stem cells. FGF-signalling is involved in different processes, including regulation of cell proliferation and epithelial-to-mesenchymal transition (EMT). These processes play an important role during reprogramming. In this study, we aimed to elucidate the role of Fgf4 during the early phases of reprogramming. We could identify Fgf4 as an essential key player regulating cell proliferation and inducing mesenchymal-to-epithelial transition (MET) through the upregulation of epithelial genes and downregulation of mesenchymal genes.

Material and Methods

Cell culture and reprogramming

For primary iPSC induction, mouse embryonic fibroblasts (MEF) were isolated from 14.5 day-pregnant C57BL/6 mice and cultured in DMEM supplemented with 10% FBS (PAA) and 1% L-glutamin/penicillin/streptomycin (10,000 U/ml penicillin G sodium; 10,000µg/ml streptomycin sulphate; 29.2mg/ml L-glutamine; 10mM sodium citrate in 0.14% NaCl, Gibco). The reprogramming of the MEFs was performed according to Yamanaka's protocol [10] using the pMXs retroviral vectors producing murine Oct4, Sox2, Klf4 and c-Myc (Addgene, cat. nos. 13366, 13367, 13370 and 13375). Two days after infection, MEFs were cultured in DMEM containing 15% FBS, 1% L-glutamin/penicillin/streptomycin, 1x MEM non-essential amino acids (GIBCO), 100 mM sodium pyruvate (Sigma) and 50 mM ß-mercaptoethanol (GIBCO) supplemented with 1000 U/ml ESGRO murine Leukemia inhibitory factor (LIF, Chemicon Int.) (Referred to as iPSC culture medium).

For secondary iPSC induction, MEFs containing a single doxycycline-inducible polycistronic transgene (2nd MEFs) expressing the four reprogramming factors from the collagen type 1 locus were used [11]. Secondary MEFs were seeded at 30-40 cells x mm^{-2} and eight hours after plating, the medium was changed to iPSC culture medium supplemented with 1ug/ml doxycycline (iPSC culture medium + Dox). After 4 days of induction, the 2nd MEFs were reseeded at 60-65 cells x mm^{-2} on mitomycin C-treated MEFs in iPSC culture medium + Dox. After ESC-like colonies appeared, the colonies were mechanically picked up and digested for culturing on mitomycin C-treated MEF cells in iPSC culture me-

dium without doxycycline. Once the iPSC colonies were picked, none of the small chemical compounds were added to the medium during long-term culture, unless otherwise stated.

FGF4 (Sigma), PD173074 (Calbiochem) and SU5402 (Calbiochem) were used at concentrations of 10ng/ml, 100ng/ml and 2μM, respectively. The Alk-4/5/7 inhibitor, A83-01 (Stemgent), was used at a concentration of 0.5uM. The anti-human FGF4 antibody (R&D System, Inc.) was used at a concentration of 100ng/ml.

Colony counting assay

To investigate the effect of the FGF- and TGFβ-pathway on iPSC induction, 2nd MEFs were treated with FGF4, PD173074, SD-208, A83-01 or anti-human FGF4 antibody at different time points and in various combinations. The efficiency of iPSC generation was determined by counting the number of colonies that were both alkaline phosphatase (AP) positive and morphologically resembled ESC colonies. AP staining was performed with the Alkaline Phosphatase Staining Kit (Stemgent) according to the manufacturer's instructions. AP staining was applied to the cells at reprogramming day 14-16.

Quantitative Real-time PCR

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN) and one microgram of total RNA was reverse transcribed with Oligo-dT primers (Invitrogen) and Superscript III (Invitrogen). Real-time PCR was performed in triplicates in a Rotor-Gene Q RG-6000 (QIAGEN) with Rotor-Gene SYBR green (QIAGEN) and analyzed with the Delta Ct-method. GAPDH was used for normalization. Error bars represent the standard deviation of the mean of triplicate reactions. Primers are listed in Supplemental Table 1.

Immunofluorescence staining iPSCs

For immunofluorescence staining, iPSCs derived from 2nd MEFs in the presence or absence of FGF4 or PD173074 were grown on mitomycin C-treated MEFs and fixed in 4% paraformaldehyde. Then, iPSCs were incubated with primary antibodies against Nanog (mouse anti Nanog, ReproCELL), Oct4 (rabbit anti Oct4, Santa Cruz Biotechnology) and SSEA-1 (mouse anti SSEA1, Millipore). Secondary fluorescence-labelled antibodies were used for detection (goat anti-rabbit Alexa Fluor 594 and goat anti-mouse Alexa Fluor 488, Molecular Probes, Invitrogen). Nuclei of the cells were counterstained with DAPI (Roche, Basel, Switzerland).

***In vitro* differentiation**

For monoculture neural and smooth muscle differentiation, iPSCs were plated onto gelatinized 35mm dishes. The iPSCs were cultivated for 10 days with neural differentiation medium (DMEM/F12 (GIBCO), N2 (1:100, GIBCO), B27 (1:50, GIBCO) and 1% L-glutamin/ penicillin/streptomycin) or smooth muscle differentiation medium (DMEM and 10% FBS). At day 10, cells were fixed in 4% paraformaldehyde and stained for βIII-tubulin (mouse anti βIII-tubulin isotype III, Sigma) and smooth muscle actin (mouse anti smooth muscle actin, Sigma), respectively. Secondary fluorescence-labelled

antibodies were used for detection (donkey anti-mouse Alexa Fluor 594 and goat anti-mouse Alexa Fluor 488, Molecular Probes, Invitrogen). Nuclei of the cells were counterstained with DAPI (Roche, Basel, Switzerland).

Microarray Analysis

Total RNA was extracted from retroviral reprogrammed MEFs treated with or without 100ng/ml PD173074 for 4 days using the RNeasy Mini Kit. Samples of both treatments were prepared in four biological replicates. Affymetrix Mouse Gene 1.1 ST Array (Affymetrix) was used and all experiments were performed at the Functional Genomics Centre Zurich.

Results

Activation of FGF receptor tyrosine kinase activity by Fgf4 is essential for the reprogramming process

We have recently shown that Parp1 mediated ADP-ribosylation of Sox2 is essential for the activation of *Fgf4* expression and that Fgf4 is essential for the initiation of the reprogramming process [3]. To further determine the role of Fgf4, MEFs derived from C57BL/6 mice were transduced with retroviruses producing OSKM (Oct4, Sox2, Klf4 and c-Myc) transcription factors. During reprogramming, the MEFs were treated with the small-chemical inhibitors SU5402 [12] or PD173074 (PD17) [13] to block the Fgf receptor (FgfR) tyrosine kinase activity. Addition of SU5402 during reprogramming reduced the number of iPSC colonies of about 50% (Fig. 1A), which is in accordance with our previously published study [3]. To confirm that blockade of FGF-signalling is the critical target of SU5402, we used the alternative inhibitor PD17. Also in this case the number of iPSC colonies was reduced by around 70% compared to untreated (Ctrl) WT fibroblasts (Fig. 1A). The higher reduction in the WT cells treated with PD17 is consistent with its higher affinity for the Fgf receptor. We therefore used PD17 for further experiments in this study.

In order to investigate the molecular mechanism underlying the reduction of reprogramming efficiency by PD17, we performed a microarray analysis. We compared global gene expression changes occurring at day 4 upon retrovirus-mediated transduction of MEFs in the presence or absence of PD17. Cluster analysis of transcriptionally regulated genes indicated that genes controlling EMT and cell cycle progression were significantly differentially expressed (Fig. 1B). To obtain an overview on the most representative biological processes we gathered the information from the GeneGo pathways and investigated similarities and differences locally, within the context of pathways and biological processes. This comparison confirmed that inhibition of the FGF pathway during iPSC induction significantly reduces the efficiency by affecting genes related to EMT/MET (Fig. 1C and 1D).

Generation of iPSCs by primary infection using viral gene delivery results in heterogeneous cell populations due to proviral integrations that vary in both number and genomic location. Secondary sys-

tems based on doxycycline (Dox) inducible lentiviruses or transgenes can be reactivated in somatic cells derived from primary iPSCs [11, 14-16]. This system allows studying reprogramming in homogenous populations of cells. Thus, to further confirm the effect of the FGF pathway on reprogramming, we used mouse secondary MEFs (2nd MEFs) carrying a single Dox-inducible polycistronic cassette harbouring all four reprogramming factors targeted to the inert ColA1 (collagen type I) locus [11].

In a first attempt, 2nd MEFs were reprogrammed upon Dox induction in the presence of PD17 or FGF4. The number of iPSC colonies was assessed by alkaline phosphatase (AP) staining 14 days after induction. Confirming our previous observations, significantly less AP-positive colonies were detected in the fibroblasts treated with PD17 compared to the untreated 2nd MEFs (Ctrl) (Fig. 2A). In contrast, the number of AP-positive colonies in the FGF4 treatment group was significantly higher than the control group (Fig. 2A). To validate that the supportive effect of FGF4 is mediated through the activation of the FgfR tyrosine kinases, 2nd MEFs were reprogrammed in the presence of PD17 and FGF4. The number of colonies obtained was comparable to level of the PD17 treated cells (Fig. 2A) clearly demonstrating that the FGF pathway plays a striking role during the reprogramming process.

It is known that various members of the FGF family regulate FGF-signalling [17]. To further test the importance of Fgf4, we reprogrammed 2nd MEFs in the presence of neutralizing anti-FGF4 (a-FGF4) antibody. The resulting decrease in the number of AP-positive colonies compared to the Ctrl confirms that Fgf4 is crucial for the efficient generation of iPSCs (Supplemental 1A).

To investigate whether differential expression of the gene *Fgf4* influenced reprogramming efficiency, we harvested mRNA during the reprogramming process and analysed the expression level of *Fgf4*. We found that the expression of *Fgf4* in all three conditions (Ctrl, PD17 and FGF4) gradually increased during the reprogramming and reached a plateau at the end of the process (Fig. 2B). The continual increase in the *Fgf4* expression is accelerated in cells treated with FGF4 and delayed in PD17 treated cells. However, a significant difference between Ctrl and FGF4 treated cells was only detectable at d4 and d8 and between Ctrl and PD17 treated cells at d10. This experiment suggests that endogenous *Fgf4* expression is not influenced by the addition of either PD17 or FGF4.

In summary, our data confirm that Fgf4-mediated activation of the FgfR tyrosine kinases is crucial for an efficient reprogramming process no matter if a conventional retrovirus based reprogramming approach or the secondary inducible system is used. Furthermore, supplementation of FGF4 during iPSCs induction can significantly increase the reprogramming efficiency.

We assessed the pluripotent state of the iPSC colonies obtained from the reprogrammed 2nd MEFs in the presence of PD17 or FGF4 by picking the colonies on days 14-16 after Dox addition. Picked iPSCs were passaged in medium without Dox, PD17, or FGF4. All iPSCs lines tested expressed the pluripotency markers Oct4, SSEA-1, Sox2, Nanog and Rex1 (Fig. 3A and 3B) and further, were able to differentiate into smooth muscle and neuronal progenitor cells *in vitro* (Fig. 3C). Taken together,

these results demonstrate that the iPSCs generated in the presence of PD17, despite the very low efficiency during the reprogramming process, or FGF4 are pluripotent.

Dissecting the role of Fgf4 in the different phases of the reprogramming process

Several studies have shown that the reprogramming process can be divided into three phases – initiation, maturation and stabilization (reviewed in [18]). We sought to investigate during which period of the reprogramming process FGF-signalling is important. Therefore, we segmented the reprogramming process into three phases (d0-d4 / d4-d8 / d8-d12). To this end, 2nd MEFs were reprogrammed and cultivated in medium containing PD17 either between d0-d4, d4-d8, or d8-d12. As a control untreated 2nd MEFs and 2nd MEFs cultivated with PD17 during the entire process were used. Fourteen days after the induction, the resulting iPSCs colonies were stained for AP-activity and positive colonies were counted. As shown in Figure 4A, addition of PD17 caused a significant reduction of the reprogramming efficiency compared to the Ctrl. This decrease was independent from the phase of the reprogramming process. Remarkably, the addition of PD17 during d4-d8 resulted in a massive reduction of AP-positive colonies compared to the control treated with PD17 (Fig. 4B). These results clearly indicate that inhibition of the FGFR tyrosine kinases activity is adverse in every phase of the reprogramming process. However, the strongest impact on the reprogramming efficiency was observed when the FGF-signalling was inhibited during d4-d8.

To more specifically analyse the role of Fgf4 in the three phases, we repeated the reprogramming of 2nd MEFs in the presence of neutralizing a-FGF4 antibody. Comparison of the AP-positive colonies in the presence of a-FGF4 antibody with the untreated Ctrl revealed a reduction of the reprogramming efficiency in all three reprogramming phases (Supplemental 1B). As depicted in Figure 4C, and in accordance with the previous data with PD17, the neutralization of Fgf4 during d4-d8 caused the most significant decrease in the reprogramming efficiency. These results suggest that the ability of Fgf4 to activate FGF-signalling is important throughout the whole reprogramming process, but is most crucial between d4-d8.

We were further interested whether the positive effect of FGF4 (see Figure 2A) on the reprogramming efficiency is phase dependent. Therefore, 2nd MEFs were reprogrammed in the presence of FGF4 during the three phases of reprogramming as described in the previous experiments. The resulting iPSC colonies were stained for AP-activity and counted. Supplementation with FGF4 during d0-d4 had no effect, whereas addition of FGF4 during d4-d8 led to a significantly increased reprogramming efficiency. Interestingly, a similar increase in efficiency was observed when 2nd MEFS were treated with FGF4 during the entire process. Finally, addition of FGF4 during the last phase of the reprogramming process (d8-d12) had a negative impact on the amount of AP-positive colonies (Fig. 4D).

Taken together, these experiments clearly show that the activation of the FGF-signalling between d4-d8 by Fgf4 is crucial for an efficient reprogramming process. Remarkably, complementation of the medium with additional FGF4 improves the reprogramming efficiency in this phase.

In the early phases of reprogramming, Fgf4 controls cell proliferation and mediates mesenchymal-to-epithelial transition

To closer investigate the role of FGF-signalling, we analysed processes regulating the reprogramming process in which FGF-signalling might potentially be involved. One process regulating reprogramming is proliferation. It was previously shown that proliferation is needed for the acquisition of stochastic epigenetic changes during reprogramming [19]. Since Fgf4 is able to accelerate cell proliferation [20], we asked whether Fgf4 increases the cellular proliferation rate during reprogramming. To achieve this, gene expression profiles of several cell cycle-related genes ($p15^{Ink4b}$, $p16^{Ink4a}$, $p19^{Arf}$) were analysed in 2nd MEFs treated with PD17 or FGF4. In comparison to the control group (untreated 2nd MEFs), the expression of all three genes was downregulated in the FGF4 treated group. In contrast, $p15^{Ink4b}$ and $p19^{Arf}$ were found to be upregulated in the 2nd MEFs treated with PD17 (Fig. 5A). These results indicate that FGF-signalling plays a role in the regulation of the cellular proliferation during reprogramming and that Fgf4 is able to down-regulate the expression of cell cycle inhibitory genes.

It has been shown that the induction of MET is an early requisite event during reprogramming of MEFs into iPSCs [21, 22]. As demonstrated in our transcriptomic analysis (Fig. 1B), the addition of PD17 during reprogramming affects a number of genes involved in EMT/MET. We therefore concentrated our analyses on the expression of genes related to MET during reprogramming. To this end, we isolated mRNA of untreated 2nd MEFs (Ctrl) and 2nd MEFs treated with either PD17 or FGF4 during the first 8 days of the reprogramming process. We then analysed expression changes of epithelial-associated genes, including cell-cell adhesion molecules and cytokeratins (E-cadherin/ E-cad, epithelial cell adhesion molecule/ Ep-Cam, Claudin-3/ Cldn3, keratin 8/ Krt8 and Occludin/ Ocln), and mesenchymal-associated genes, including zinc finger transcription factors and cell adhesion molecules (snail homolog 1/ Snail, snail homolog 2/ Slug, zinc finger E-box binding homeobox 2/ Zeb2, cadherin 2/ N-cad and fibronectin 1/ FN). The expression levels of several epithelial genes were upregulated in FGF4 treated 2nd MEFs when compared to the expression levels in the untreated 2nd MEFs (Ctrl). On the contrary, the presence of PD17 did not affect the expression levels of the epithelial genes (Fig. 5B). Analysis of mesenchymal associated genes revealed that addition of FGF4 downregulated the expression levels of several genes compared to the Ctrl. In contrast, presence of PD17 during the reprogramming process caused an upregulation of the mesenchymal-related genes Zeb2, Snail and FN (Fig. 5C). Taken together, these data indicated that addition of FGF4 favours MET by

upregulating the expression of epithelial genes and downregulating the expression of mesenchymal genes. Conversely, this process is impaired by the addition of PD17.

Blocking of TGFβ pathways only partially restores the need for Fgf4

Based on the discovery that Fgf4 promotes the activation of the MET program, we wondered if a crosstalk with other pathways is involved as well. Notably, the zinc-finger transcription factors Snail and Zeb2 are known to maintain the mesenchymal phenotype by directly repressing epithelial gene expression [23]. The activation of Snail occurs downstream of TGFβ-signalling, a well known EMT inducer [24, 25]. We therefore tested whether the inhibition of the TGFβ pathway and the subsequent suppression of EMT can rescue the observed negative effect of PD17 during reprogramming of 2nd MEFs. To this end, we reprogrammed 2nd MEFs in the presence of PD17 and A83-01, an inhibitor of the type 1 Tgfβ receptor ALK5, which blocks the phosphorylation of Smad2 and thus inhibits Tgfβ-induced EMT [26, 27]. As shown in Figure 6A, the combination of A83-01 and PD17 during the complete reprogramming process significantly increased the efficiency of iPSC colony formation when comparing to 2nd MEFs treated with PD17 alone. To specify during which period of the reprogramming process inhibition of TGFβ-signalling by A83-01 exerts a positive effect in rescuing the PD17-phenotype (see Fig. 6A) we repeated the reprogramming experiment adding both PD17 and A83-01 between d0-d4, d4-d8 or d8-d12. The addition of A83-01 to PD17 treated cells between d0-d4 and d8-d12 increased the reprogramming efficiency to a level comparable to 2nd MEF controls (Fig. 6B). Interestingly, the inhibition of the TGFβ pathway between d4-d8 was not sufficient to completely rescue the PD17 phenotype and lead to a decrease in the reprogramming efficiency (Fig. 6B).
Altogether, these results clearly demonstrate that inhibition of FGF-signalling and the resulting negative effect on MET can be compensated in the early and late phase of reprogramming by blocking TGFβ-signalling. Nevertheless, inhibition of FGF-signalling in the central phase of the reprogramming process (d4-d8) cannot be compensated by the addition of A83-01, enlightening the importance of Fgf4 to drive MET in the central phase of the reprogramming process.

Discussion

Based on our recent findings stating that poly(ADP-ribosylation) of Sox2 by Parp1 plays an important role during the first days of the reprogramming process through regulation of *Fgf4* expression [3], we in this study aimed to better characterize the role of Fgf4 during reprogramming. In a first step, we inhibited FgfR tyrosine kinase activity by adding small chemical inhibitors (SU5402 or PD173074). In accordance with our previous observations [3], treatment with the inhibitors massively reduced the number of AP-positive iPSC colonies.

Generation of iPSCs by primary infection using viral gene delivery results in heterogeneous cell populations due to proviral integrations that vary in both number and genomic location. In order to reduce the variability linked to this system we opted to use a secondary system based on a doxycycline (Dox) inducible transgenic system that can be reactivated in somatic cells derived from primary iPSCs. Analysis of the reprogramming process in 2nd MEFs indicated that the observed effects were to ascribe to Fgf4 and not to other members of the Fgf family. This is also corroborated by the fact that 2nd MEFs, reprogrammed in the presence of neutralizing a-FGF4 antibody, showed a decrease in the number of AP-positive colonies compared to the controls (Supplemental Figure 1A), comparable to the decrease observed in presence of the inhibitor (Figure 2A). Furthermore, when FGF4 was added in presence of the PD17 inhibitor, the reprogramming efficiency was still strongly reduced compared to controls. Taken together, this data clearly indicate that FGF4 promotes reprogramming through the activation of the FgfR tyrosine kinases.

Based on the previous knowledge that the reprogramming process can be divided into the three phases – initiation, maturation and stabilization – we wondered if FGF-signalling is important only during a specific period or during the entire process. To test this, we applied the PD17 inhibitor during different time windows: d0-d4, d4-d8 and d8-d12. Our results revealed that inhibition of the FgfR tyrosine kinases activity is adverse during all three phases of the reprogramming process. The strongest impact can be observed during d4-d8 (Fig. 6CI). These observations were also confirmed by using a neutralizing a-FGF4 antibody. Also in this case, the most significant decrease in efficiency was observed between d4-d8 (Fig. 6CIII).

Fgf4 is known to be expressed in ESCs and iPSCs rather than in fibroblasts. The early activation of *Fgf4* expression observed in our previous studies [3] and in the present work (Fig. 2B) clearly indicate that Fgf4 might have a specific role during the first phases of reprogramming. Fgf4 could regulate the proliferation rate, because it is able to accelerate cell proliferation [20]. Furthermore, previous studies showed that proliferation is needed for the acquisition of stochastic epigenetic changes during reprogramming [19]. We monitored the gene expression profiles of several cell cycle-related genes ($p15^{Ink4b}$, $p16^{Ink4a}$, $p19^{Arf}$) in 2nd MEFs treated with PD17 or FGF4 and found that all three genes were downregulated in the FGF4 treated group and that $p15^{Ink4b}$ and $p19^{Arf}$ were upregulated in presence of PD17 (Fig. 5A). These results indicate that FGF-signalling indeed plays a role in the regulation of cell proliferation during reprogramming. Moreover, FGF4 is able to down regulate the expression of cell cycle inhibitory genes.

By comparing fibroblasts treated with or without PD17 in the first days of reprogramming, our transcriptomic analysis indicated that a number of genes involved in the process of EMT were significantly differentially expressed. During development, EMT is essential for the proper formation of the body plan and the differentiation into many tissues and organs. During this process epithelia convert

to mesenchyme through multiple rounds of EMT accompanied by the reversible process of MET [28]. In adult tissues, the EMT program is reactivated during specific processes like wound healing, organ fibrosis and tumour progression [29]. EMT/MET play pivotal roles not only during organ development, but also in cancer metastasis by providing cells with migratory and invasive properties [23]. During these processes, critical developmental signalling pathways like Wnt, Notch, Hedgehog and Tgfβ are reactivated. Of note, during reprogramming, fibroblasts gradually lose their differentiated identity and acquire ESC gene expression patterns and growth behaviour associated to pluripotency. Morphologically, this conversion is linked to a transition from a single layer of adherent cells to a multilayer of epithelial cells; a process that strongly resembles MET. Recent studies indeed confirmed that MET is a crucial process during the early reprogramming of murine embryonic fibroblasts into iPSCs [21, 22]. These works revealed that in the earliest phases of the reprogramming process fibroblasts undergo changes in gene expression, typical for MET, like upregulation of epithelial genes such as E-cadherin and Ep-Cam as well as parallel downregulation of mesenchymal genes such as Snail and N-cadherin. These studies identified members of the Tgfβ superfamily such as Smads and BMP receptors as essential key players for iPSC formation [22]. Furthermore, these observations are corroborated by the fact that the treatment of fibroblasts with Tgfβ inhibitors enhances iPSC formation [30-32]. Of note, during embryo development EMT/MET are triggered not only by members of the Tgfβ family, but also by components of the extracellular matrix, such as collagen and hyaluronic acid, as well as soluble growth factors, like members of the FGF and epidermal growth factor (EGF) families. We therefore decided to analyze the expression changes of EMT/MET-related genes during the different phases of reprogramming. We isolated RNA from untreated 2nd MEFs and from 2nd MEFs treated with either PD17 or FGF4 during the first 8d of the reprogramming process. Then, we analysed the expression changes of epithelial-associated genes, including cell-cell adhesion molecules like E-cad, Ep-Cam, Cldn3, Krt8 and Ocln, and mesenchymal-associated genes, including Snail, Slug, Zeb2, N-cad and FN. Expression levels of several epithelial genes were higher in FGF4 treated 2nd MEFs than in untreated 2nd MEFs. In contrast, the presence of PD17 did not affect the expression levels of the epithelial genes (Fig. 5B). Exogenous addition of FGF4 induced a downregulation of mesenchymal associated genes, whereas treatment with PD17 caused an upregulation of the mesenchymal-related genes Zeb2, Snail and FN (Fig. 5C). In summary, our data indicate that during the first phases of reprogramming the presence of Fgf4 favours MET by the parallel upregulation of epithelial gene expression and downregulation of mesenchymal genes. This process is impaired by PD17. These observations are in agreement with previous reports describing that MET-associated alterations were evident during the initiation phase of reprogramming, when cells are still dependent on exogenous factor expression. During the subsequent maturation and stabilization phases, cells activate an ESC-like gene expression program and the exogenous reprogramming factors are silenced [22].

Our data raise the question if the observed effects are the result of a crosstalk between FGF pathway and other pathways known to be involved in MET. Notably, the zinc-finger transcription factors Snail and Zeb2 are known to support the mesenchymal phenotype through direct repression of epithelial genes [23]. Activation of Snail occurs downstream of TGFβ-signalling [24, 25]. We asked whether suppression of EMT through inhibition of the TGFβ pathway could rescue the observed negative effect of PD17 during reprogramming. To this end we reprogrammed 2nd MEFs in the presence of both PD17 and A83-01 and detected a significantly higher efficiency of iPSC colony formation than in cells treated with PD17 alone (Fig. 6A). This rescuing effect was most prominent between d0-d4 and d8-d12 where reprogramming efficiencies comparable to the 2nd MEF controls were obtained (Fig. 6B). Interestingly, inhibition of the TGFβ pathway between d4-d8 was not sufficient to completely rescue the PD17 phenotype. These findings clearly demonstrate that inhibition of the FGF-signalling and the resulting negative effect on MET can be compensated by blocking TGFβ-signalling in the early and late phase of reprogramming. However, this is not possible in the central phase (d4-d8), enlightening the importance of Fgf4 to drive MET in the central phase of the reprogramming process (Fig. 6CIV).

Conclusion

Our work for the first time identifies Fgf4 as an important soluble factor for promoting the central phase (d4-d8) of reprogramming (Fig. 6CII). In doing so, Fgf4 regulates cell proliferation and favours MET via parallel upregulation of epithelial gene expression and downregulation of mesenchymal genes.

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Author Disclosure Statement

All authors state that they have no competing financial interests.

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Figure legends

Figure 1:

Inhibition of the FgfR tyrosine kinases activity reduces the reprogramming efficiency and affects EMT/MET A) Inhibition of the FgfR tyrosine kinases activity reduces reprogramming efficiency. WT fibroblasts were reprogrammed using retroviruses in the presence of SU5402 and PD17. The number of iPSCs colonies was assessed for the untreated cells (Ctrl) and the fibroblasts treated with SU5402 or PD17 at day 16. The data shown represent three independent repeats and mean values + standard deviation (SD) are depicted. Statistical values were calculated using Dunnett's multiple comparison test. ****p<0.0001. B) Heat map of mRNA expression on fibroblasts treated with PD17 at day 4 upon starting of reprogramming. The signature used in the heat map was compiled from Affymetrix probe sets related to genes involved in EMT/MET (88 probe sets), cell cycle (23 probe sets) and metabolism (32 probe sets). C) Most significantly enriched Gene Ontology Biological Process terms according to GeneGo. D) Most significantly enriched Gene Ontology Maps terms according to GeneGo.

Figure 2:

Activation of the FGF pathway can increase the reprogramming efficiency. A) Secondary MEFs were reprogrammed in the presence of PD17, FGF4 or PD17 + FGF4. Untreated, reprogrammed 2nd MEFs served as control. The efficiency was assessed by the number of AP-positive colonies in the different treatment groups compared to the untreated Ctrl. The data shown represent three independent repeats and the mean values + SD are depicted. Statistical values were calculated using Dunnett's multiple comparison test. ****p<0.0001. B) Secondary MEFs were treated for the entire reprogramming process with PD17 or FGF4. *Fgf4* expression levels were analysed by RT-qPCR for the indicated days in the different treatment groups and normalized to the expression level in the WT cells (Ctrl) at d14. Samples were measured in triplicates and the mean values + SD are shown. Statistical values were calculated using Student's t-test. *p<0.05, **p<0.01. n.d. = not detected.

Figure 3:

Characterization of WT (Ctrl) iPSCs and iPSCs generated with FGF4 or PD17 during the reprogramming process. A) Immunostaining for Nanog, Oct4 and SSEA1 in the different iPSC lines. The scale bars represent 100um. B) Expression for the pluripotency genes Oct4, Rex1, Nanog and Sox2 were determined by RT-qPCR analysis of the different iPSC lines as indicated. The expression levels were normalized to the WT iPSC line #1. Samples were measured in triplicates and the mean values + SD are shown. C) Immunostaining for smooth muscle actin (SMA) and βIII-tubulin for *in vitro* differentiated iPSC lines. The scale bars represent 100µm.

Figure 4:

The FGF-signalling between d4-d8 is crucial for an efficient reprogramming process. A) Secondary MEFs were reprogrammed and PD17 was added during three different time frames: d0-d4, d4-d8 and d8-d12. Untreated 2nd MEFs and 2nd MEFs treated during the entire reprogramming process with PD17 served as controls. B) The reprogramming efficiency of 2nd MEFs treated during the entire reprogramming with PD17 was compared to 2nd MEFs treated during a defined time frame with PD17. C) The reprogramming efficiency of 2nd MEFs treated during the entire process with anti-FGF4 (a-FGF4) antibody was compared to 2nd MEFs treated during a defined time frame with a-FGF4 antibody. D) Secondary MEFs were reprogrammed and FGF4 was added during three different time frames: d0-d4, d4-d8 and d8-d12. Untreated 2nd MEFs and 2nd MEFs treated during the entire reprogramming process with FGF4 served as controls. All the data shown represent three independent repeats and the mean values + SD are depicted. Statistical values were calculated using Dunnett's multiple comparison test. Ns = not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 5:

FGF-signalling influences cellular proliferation and MET. A) Expression of $p15^{Ink4b}$, $p16^{Ink4a}$ and $p19^{Arf}$ was determined by RT-qPCR in 2nd MEFs and on day 2, 4, 6 and 8 during reprogramming in the presence or absence of PD17 or FGF4. B) RT-qPCR for epithelial-related genes was performed for the indicated time points and conditions. C) RT-qPCR for mesenchymal-related genes was performed for the indicated time points and conditions. All samples shown in this figure were measured in triplicates and the mean values + SD are shown.

Figure 6:

FGF-signalling is crucial during d4-d8 of the reprogramming process for inducing MET. A) Secondary MEFs were reprogrammed in the presence of PD17, A83-01 or PD17 + A83-01. Untreated, reprogrammed 2nd MEFs served as control. The efficiency was assessed by the number of AP-positive colonies in the different treatment groups compared to the untreated Ctrl. B) Secondary MEFs were reprogrammed and PD17 + A83-01 was added during three different time frames: d0-d4, d4-d8 and d8-d12. Untreated 2nd MEFs and 2nd MEFs treated during the entire reprogramming process with PD17, A83-01 or PD17 + A83-01 served as controls. C) The reprogramming process requires activation of MET in the early phases and suppression of EMT for a successful and efficient reprogramming process. FGF-signalling is involved in the regulation of MET, whereas TGFβ-signalling promotes EMT. I) Inhibition of the FgfR tyrosine kinases activity by PD17 is adverse during all three phases of the reprogramming process, but the strongest impact can be observed during d4-d8. II) FGF4 is able to increase the reprogramming efficiency by promoting MET, if added from d4-d8. III) Neutralization of

Fgf4 by a-FGF4 antibody reduces the reprogramming efficiency throughout all phases, but most significantly between d4-d8. IV) Inhibition of FGF-signalling by PD17 and the resulting negative effect on MET can be compensated in the early and late phase by blocking TGFβ-signalling with A83-01. The addition of A83-01 is not sufficient to rescue the PD17 phenotype in the central phase (d4-d8). All data shown represent three independent repeats and the mean values + SD are depicted. Statistical values were calculated using Student's t-test (A) and Dunnett's multiple comparison test (B), respectively. Ns = not significant, ***p<0.001, ****p<0.0001.

Supplementary Data

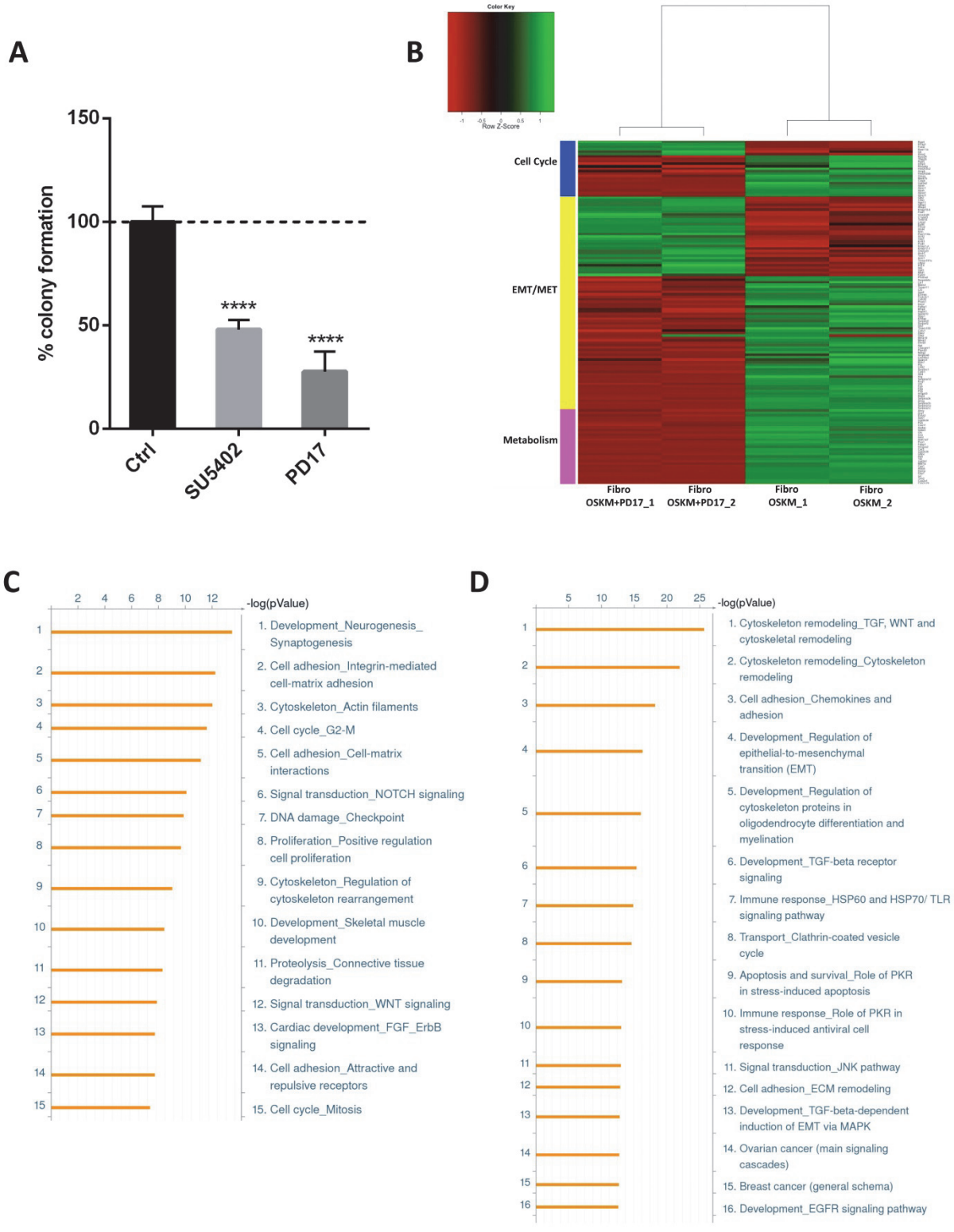
Supplemental Figure 1:

The neutralization of Fgf4 by anti-FGF4 antibody decreases the reprogramming efficiency independent of the reprogramming phases. A) Secondary MEFs were reprogrammed in the presence or absence of a-FGF4 antibody. B) Secondary MEFs were reprogrammed and a-FGF4 antibody was added during three different time frames: d0-d4, d4-d8 and d8-d12. Untreated 2nd MEFs and 2nd MEFs treated during the entire reprogramming process with a-FGF4 antibody served as controls. All the data shown represent three independent repeats and the mean values + SD are depicted. Statistical values were calculated using Student's t-test (A) and Dunnett's multiple comparison test (B), respectively. ***p<0.001, ****p<0.0001.

Supplemental Table 1:

Sequences of the primers used.

Figure 1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% colony formation
150
100
50
0
****
****
Ctrl
SU5402
PD17
B
Color Key
Row Z-Score
Cell Cycle
EMT/MET
Metabolism
Fibro OSKM+PD17_1
Fibro OSKM+PD17_2
Fibro OSKM_1
Fibro OSKM_2
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3. Cytoskeleton_Actin filaments
4. Cell cycle_G2-M
5. Cell adhesion_Cell-matrix interactions
6. Signal transduction_NOTCH signaling
7. DNA damage_Checkpoint
8. Proliferation_Positive regulation cell proliferation
9. Cytoskeleton_Regulation of cytoskeleton rearrangement
10. Development_Skeletal muscle development
11. Proteolysis_Connective tissue degradation
12. Signal transduction_WNT signaling
13. Cardiac development_FGF_ErbB signaling
14. Cell adhesion_Attractive and repulsive receptors
15. Cell cycle_Mitosis
D
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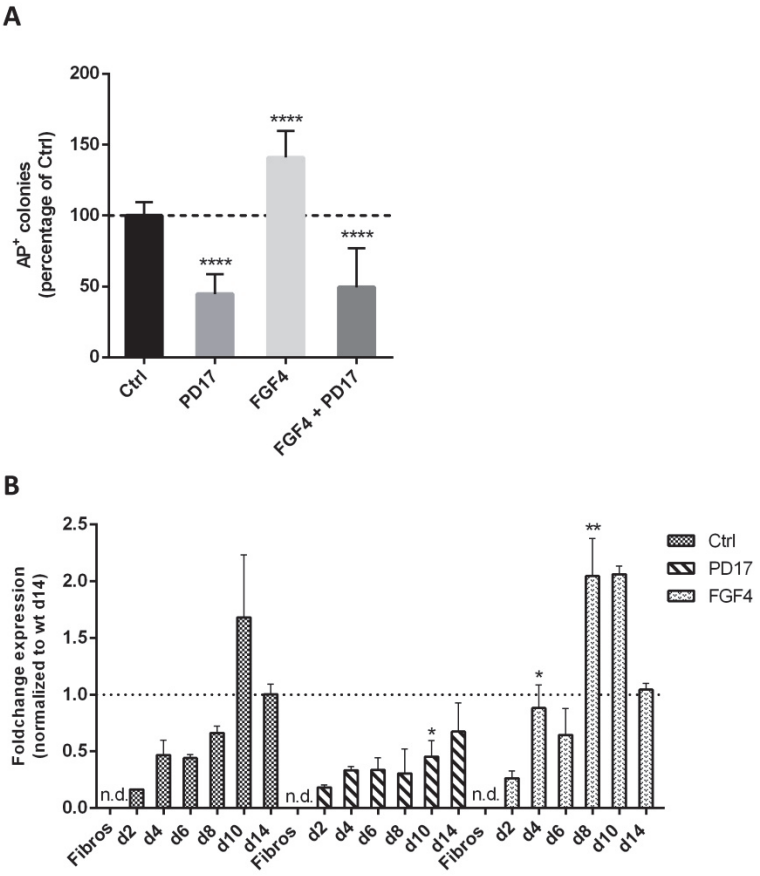
1. Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling
2. Cytoskeleton remodeling_Cytoskeleton remodeling
3. Cell adhesion_Chemokines and adhesion
4. Development_Regulation of epithelial-to-mesenchymal transition (EMT)
5. Development_Regulation of cytoskeleton proteins in oligodendrocyte differentiation and myelination
6. Development_TGF-beta receptor signaling
7. Immune response_HSP60 and HSP70/ TLR signaling pathway
8. Transport_Clathrin-coated vesicle cycle
9. Apoptosis and survival_Role of PKR in stress-induced apoptosis
10. Immune response_Role of PKR in stress-induced antiviral cell response
11. Signal transduction_JNK pathway
12. Cell adhesion_ECM remodeling
13. Development_TGF-beta-dependent induction of EMT via MAPK
14. Ovarian cancer (main signaling cascades)
15. Breast cancer (general schema)
16. Development_EGFR signaling pathway

Figure 2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AP+ colonies (percentage of Ctrl)
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150
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50
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****
****
****
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FGF4
FGF4 + PD17
B
Foldchange expression (normalized to wt d14)
2.5
2.0
1.5
1.0
0.5
0.0
n.d.
n.d.
n.d.
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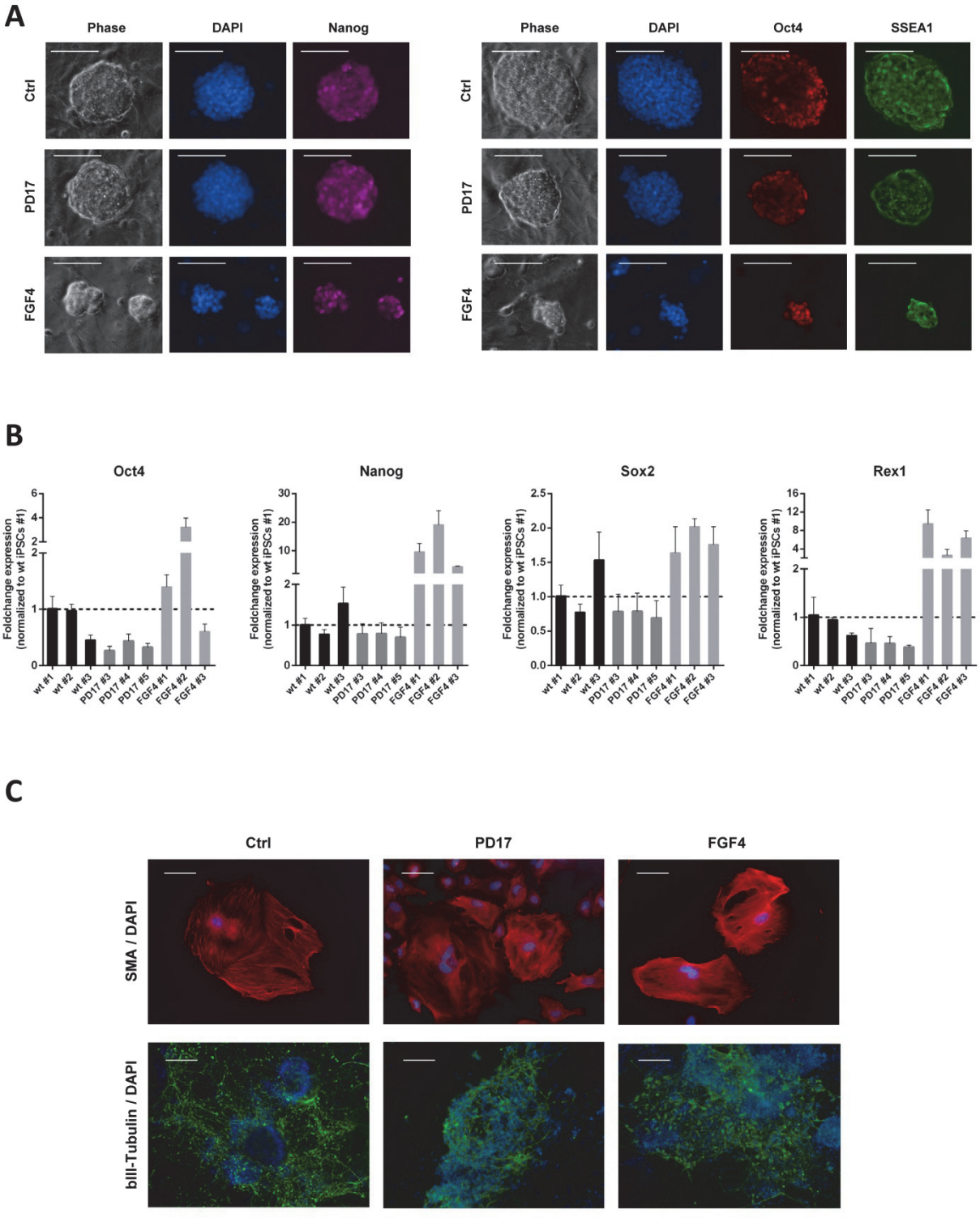
d4
d6
d8
d10
d14

Figure 3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Phase
DAPI
Nanog
Oct4
SSEA1
Ctrl
PD17
FGF4
B
Oct4
Nanog
Sox2
Rex1
Foldchange expression (normalized to wt iPSCs #1)
wt #1
wt #2
wt #3
PD17 #3
PD17 #4
PD17 #5
FGF4 #1
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Ctrl
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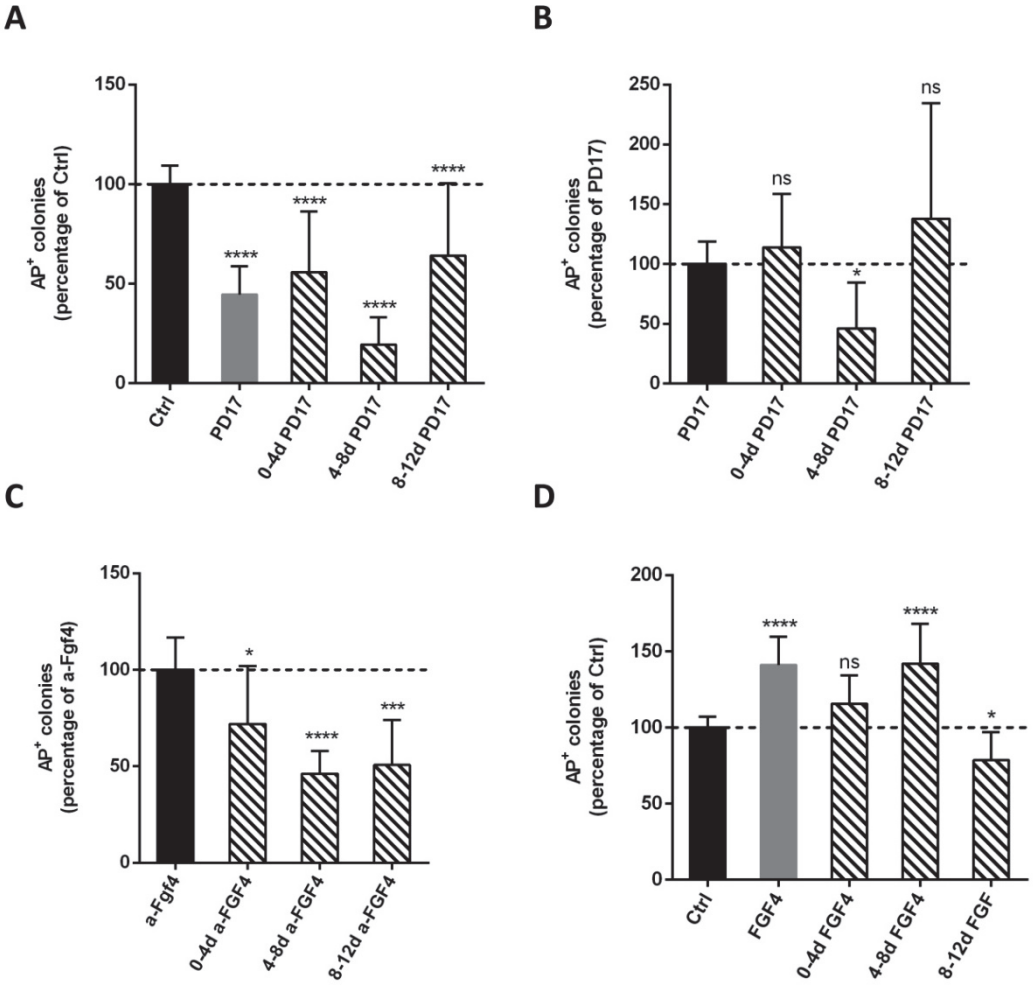
FGF4
SMA / DAPI
bIII-Tubulin / DAPI

Figure 4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C
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AP+ colonies (percentage of PD17)
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4-8d PD17
8-12d PD17
a-Fgf4
0-4d a-FGF4
4-8d a-FGF4
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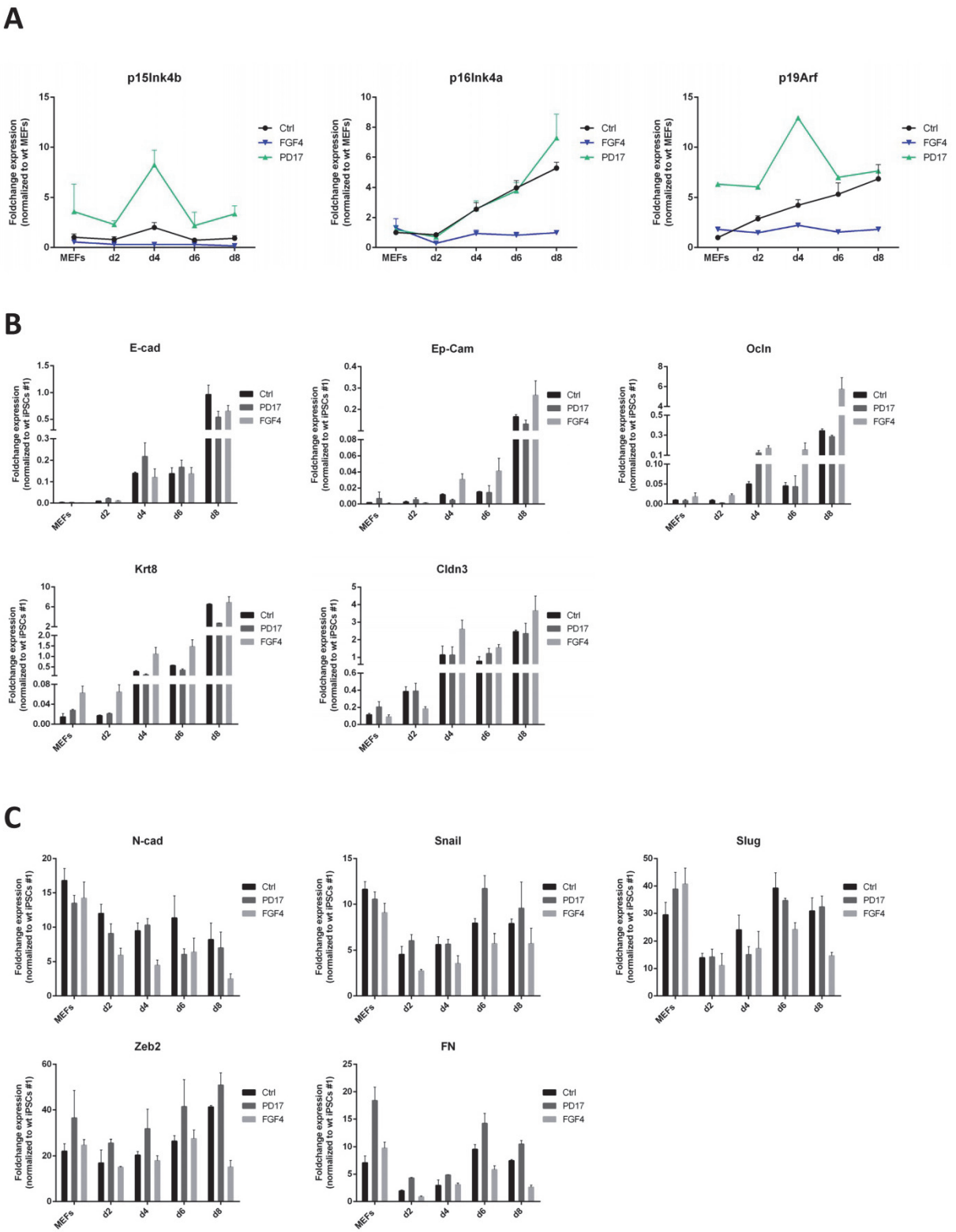
8-12d FGF
ns
****
***
*

Figure 5


A
p15Ink4b
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p19Arf
Foldchange expression (normalized to wt MEFs)
Ctrl
FGF4
PD17
MEFs
d2
d4
d6
d8
B
E-cad
Ep-Cam
Ocln
Krt8
Cldn3
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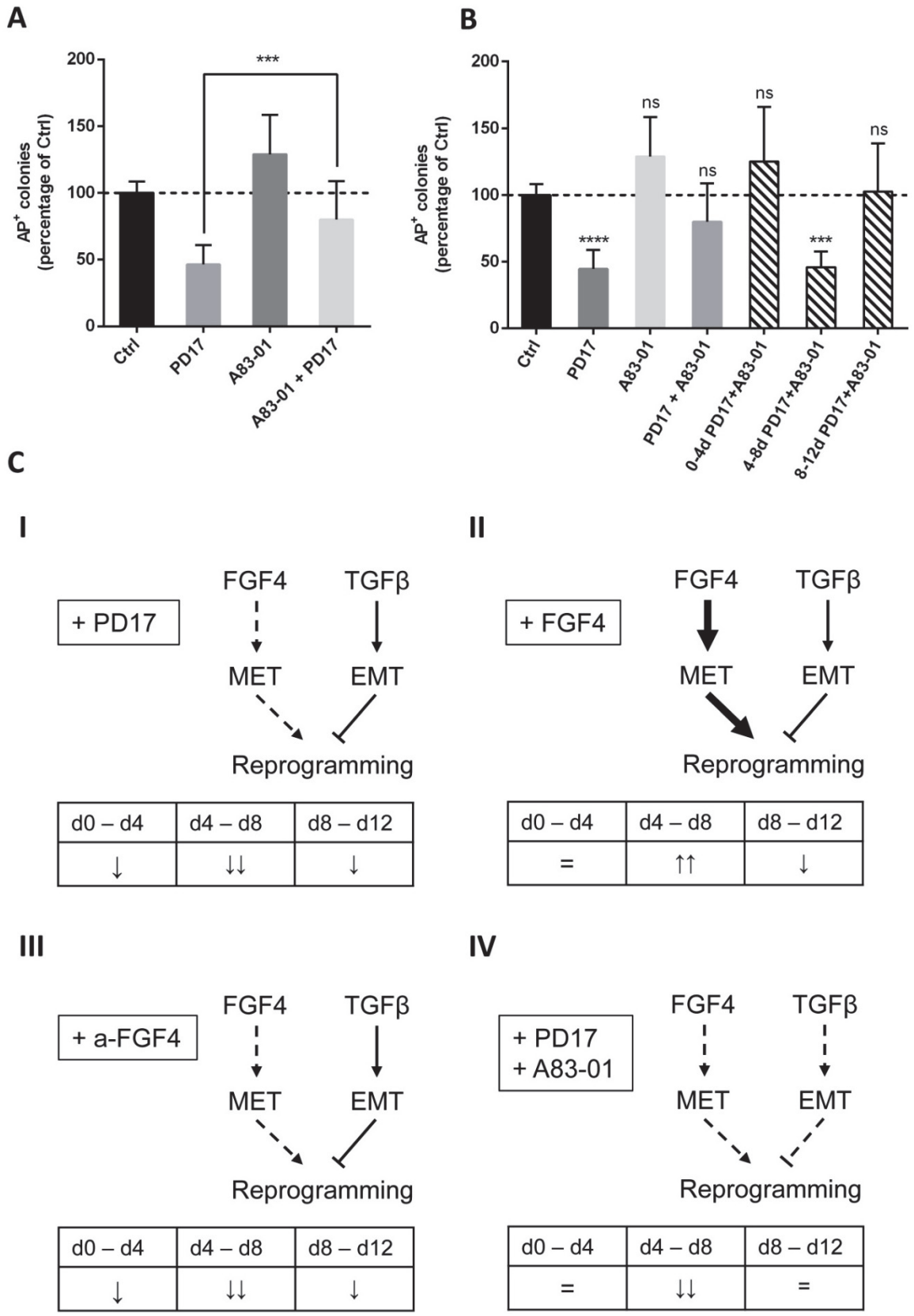
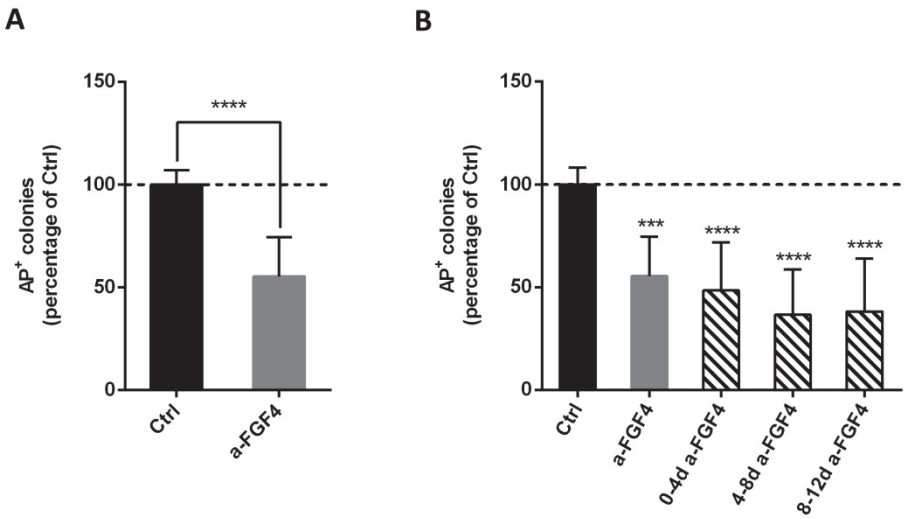
FGF4
C
N-cad
Snail
Slug
Zeb2
FN

Figure 6


A
B
C
AP+ colonies (percentage of Ctrl)
***
****
ns
Ctrl
PD17
A83-01
A83-01 + PD17
PD17 + A83-01
0-4d PD17+A83-01
4-8d PD17+A83-01
8-12d PD17+A83-01
I
II
III
IV
+ PD17
+ FGF4
+ a-FGF4
+ PD17
+ A83-01
FGF4
TGFβ
MET
EMT
Reprogramming
d0 – d4
d4 – d8
d8 – d12
↓
↓↓
=
↑↑

Supplemental Figure 1



Supplemental Table 1

GAPDH	GAPDH_FWD	5’-GCAGCGAGGCGTGGTGAGCATCTT-3’
	GAPDH_BWD	5’-CCCCTTCTTGTTCCGCCCGTTCTT-3’
Fgf4	Fgf4_FWD	5’-CGTGGTGAGCATCTTCGGAGTGG-3’
	Fgf4_BWD	5’-CCTTCTTGGTCCGCCCGTTCTTA-3’
Oct4	Oct4_FWD	5’-TCT TTC CAC CAG GCC CCC GGC TC-3’
	Oct4_BWD	5’-TGC GGG CGG ACA TGG GGA GAT CC-3’
Nanog	Nanog_FWD	5’-ACA AGG GTC TGC TAC TGA GAT GC-3’
	Nanog_BWD	5’-GGA GAC TTC TTG CAT CTG CTG G-3’
Sox2	Sox2_FWD	5’-TAG AGC TAG ACT CCG GGC GAT GA-3’
	Sox2_BWD	5’-TTG CCT TAA ACA AGA CCA CGA AA-3’
Rex1	Rex1_FWD	5’-AGA AAG CAG GAT CGC CTC AC-3’
	Rex1_BWD	5’-AGG GAA CTC GCT TCC AGA AC-3’
$p15^{Ink4b}$	$p15^{Ink4b}$_FWD	5’-AGATCCCAACGCCCTGAAC-3’
	$p15^{Ink4b}$_BWD	5’-CCCATCATCATGACCTGGATT-3’
$p16^{Ink4a}$	$p16^{Ink4a}$_FWD	5’-CGTACCCCGATTCAGGTGAT-3’
	$p16^{Ink4a}$_BWD	5’-TTGAGCAGAAGAGCTGCTACGT-3’
$p19^{Arf}$	$p19^{Arf}$_FWD	5’-GCCGCACCGGAATCCT-3’
	$p19^{Arf}$_BWD	5’-TTGAGCAGAAGAGCTGCTACGT-3’
E-cad	E-cad_FWD	5’-CAGGTCTCCTCATGGCTTTGC-3’
	E-cad_BWD	5’-CTTCCGAAAAGAAGGCTGTCC-3’
Ep-Cam	Ep-Cam_FWD	5’-GCGGCTCAGAGAGACTGTG-3’
	Ep-Cam_BWD	5’-CCAAGCATTTAGACGCCAGTTT-3’
Ocln	Ocln_FWD	5’-TTTTTGCTGTGAAAACCCGAAG-3’
	Ocln_BWD	5’-CTGTCAACTCTTTCCGCATAGT-3’
Krt8	Krt8_FWD	5’-GAATGGCCACTGAAGTCCTTG-3’
	Krt8_BWD	5’-TCCTAAGGTTGGCCAGAGGAT-3’
Cldn3	Cldn3_FWD	5’-ACCAACTGCGTACAAGACGAG-3’
	Cldn3_BWD	5’-CAGAGCCGCCAACAGGAAA-3’
N-cad	N-cad_FWD	5’-GCTGAAAATAGACCCCGTGA-3’
	N-cad_BWD	5’-TTCCTGTCCCACTCATAGGC-3’
Snail	Snail_FWD	5’-CTTGTGTCTGCACGACCTGT-3’
	Snail_BWD	5’-CTTCACATCCGAGTGGGTTT-3’
Slug	Slug_FWD	5’-ACACATTGCCTTGTGTCTGC-3’

	Slug_BWD	5’-GATGTGCCCTCAGGTTTGAT-3’
Zeb2	Zeb2_FWD	5’-ATTGCACATCAGACTTTGAGGAA-3’
	Zeb2_BWD	5’-ATAATGGCCGTGTCGCTTCG-3’
FN	FN_FWD	5’-TTCAAGTGTGATCCCCATGAAG-3’
	FN_BWD	5’-CAGGTCTACGGCAGTTGTCA-3’

Curriculum vitae

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Place of origin: Menziken AG

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10/2009	**Promotion (Dr. med. vet.) at the Vetsuisse Faculty, University of Zurich** Thesis title: *Establishment of Induced Pluripotent Stem Cells From Rat Fibroblasts* Supervisors: Prof. Kurt Bürki, PD Dr. Paolo Cinelli
10/2004 – 10/2009	**Studies of veterinary medicine, University of Zurich** Major: biomedical research
08/1998 – 06/2004	**Gymnasium St. Fidelis, Stans NW** Matura mit Schwerpunkt Physik und Angewandte Mathematik

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PUBLICATIONS:

Weber F.A., Graf U., Okoniewski M.J., Meshorer E., Cinelli P. (2014). Fgf4 Controls Mesenchymal to Epithelial Transition During the Early Phases of Reprogramming. Stem Cells, *Submitted*

Savic N., Bär D., Leone S., Weber F.A., Vollenweider E., Ferrari E., Shakova O., Cinelli P., Santoro R. (2014). The epigenetic state oft he nucleolus regulates chromatin plasticity and pluripotency of embryonic stem cells. Cell Stem Cell, *Submitted*

Weber F.A., Bartolomei G., Hottiger M.O., Cinelli P. (2013). Artd1/Parp1 regulates reprogramming by transcriptional regulation of Fgf4 via Sox2 ADP-ribosylation. Stem cells, 31, 2364–2373.

Tiaden, A.N., Breiden, M., Mirsaidi, A., Weber, F.A., Bahrenberg, G., Glanz, S., Cinelli, P., Ehrmann, M., and Richards, P.J. (2012). Human serine protease HTRA1 positively regulates osteogenesis of human bone marrow-derived mesenchymal stem cells and mineralization of differentiating bone-forming cells through the modulation of extracellular matrix protein. Stem cells, 30, 2271-2282.

Casanova E.A., Shakhova O., Patel S.S., Asner I.N., Pelczar P., Weber F.A., Graf U., Sommer L., Bürki K., Cinelli P. (2011). Pramel7 Mediates LIF/STAT3-dependent Self-renewal in Embryonic Stem Cells. Stem Cells 29(3), 474-85.